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(54) Title: GENETICALLY MODIFIED TUMOR-TARGETED BACTERIA WITH REDUCED VIRULENCE		
(57) Abstract The present invention is directed to mutant <i>Salmonella sp.</i> having a genetically modified <i>msbB</i> gene in which the mutant <i>Salmonella</i> is capable of targeting solid tumors. The invention is also directed to <i>Salmonella sp.</i> containing a genetically modified <i>msbB</i> gene as well as a genetic modification in a biosynthetic pathway gene such as the <i>purI</i> gene. The present invention further relates to the therapeutic use of the mutant <i>Salmonella</i> for growth inhibition and/or reduction in volume of solid tumors.		

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**GENETICALLY MODIFIED TUMOR-TARGETED BACTERIA
WITH REDUCED VIRULENCE**

5 This application is a continuation-in-part of
application Serial No. 08/926,636, filed September 10, 1997,
the entire disclosure of which is incorporated by reference
herein in its entirety.

10 **1. FIELD OF THE INVENTION**

The present invention is concerned with the
isolation of a gene of *Salmonella* which, when genetically
disrupted, reduces both virulence and septic shock caused by
this organism and increases sensitivity to agents which
15 promote eradication of the bacteria, e.g., chelating agents.
The nucleotide sequence of this gene and the means for its
genetic disruption are provided, and examples of the use of
tumor-targeted bacteria which possess a disruption in this
gene to inhibit growth of cancers, including, but not limited
20 to, melanoma, colon cancer, and other solid tumors are
described. The present invention also provides for the
genetic disruption of this gene in combination with
disruption of an auxotrophic gene.

25 **2. BACKGROUND OF THE INVENTION**

Citation or identification of any reference in
Section 2, or any section of this application shall not be
construed as an admission that such reference is available as
prior art to the present invention.

30 A major problem in the chemotherapy of solid tumor
cancers is delivery of therapeutic agents, such as drugs, in
sufficient concentrations to eradicate tumor cells while at
the same time minimizing damage to normal cells. Thus,
studies in many laboratories are directed toward the design
35 of biological delivery systems, such as antibodies,
cytokines, and viruses for targeted delivery of drugs, pro-
drug converting enzymes, and/or genes into tumor cells.
Houghton and Colt, 1993, New Perspectives in Cancer Diagnosis

- and Management 1: 65-70; de Palazzo, et al., 1992a, Cell. Immunol. 142:338-347; de Palazzo et al., 1992b, Cancer Res. 52: 5713-5719; Weiner, et al., 1993a, J. Immunotherapy 13:110-116; Weiner et al., 1993b, J. Immunol. 151:2877-2886;
- 5 Adams et al., 1993, Cancer Res. 53:4026-4034; Fanger et al., 1990, FASEB J. 4:2846-2849; Fanger et al., 1991, Immunol. Today 12:51-54; Segal, et al., 1991, Ann N.Y. Acad. Sci. 636:288-294; Segal et al., 1992, Immunobiology 185:390-402; Wunderlich et al., 1992; Intl. J. Clin. Lab. Res. 22:17-20;
- 10 George et al., 1994, J. Immunol. 152:1802-1811; Huston et al., 1993, Intl. Rev. Immunol. 10:195-217; Stafford et al., 1993, Cancer Res. 53:4026-4034; Haber et al., 1992, Ann. N.Y. Acad. Sci. 667:365-381; Haber, 1992, Ann. N.Y. Acad. Sci. 667: 365-381; Feloner and Rhodes, 1991, Nature 349:351-352;
- 15 Sarver and Rossi, 1993, AIDS Research & Human Retroviruses 9:483-487; Levine and Friedmann, 1993, Am. J. Dis. Child 147:1167-1176; Friedmann, 1993, Mol. Genetic Med. 3:1-32; Gilboa and Smith, 1994, Trends in Genetics 10:139-144; Saito et al., 1994, Cancer Res. 54:3516-3520; Li et al., 1994,
- 20 Blood 83:3403-3408; Vieweg et al., 1994, Cancer Res. 54:1760-1765; Lin et al., 1994, Science 265:666-669; Lu et al., 1994, Human Gene Therapy 5:203-208; Gansbacher et al., 1992, Blood 80:2817-2825; Gastl et al., 1992, Cancer Res. 52:6229-6236.

25 2.1 BACTERIAL INFECTIONS AND CANCER

Regarding bacteria and cancer, an historical review reveals a number of clinical observations in which cancers were reported to regress in patients with bacterial infections. Nauts et al., 1953, Acta Medica. Scandinavica

30 145:1-102, (Suppl. 276) state:

The treatment of cancer by injections of bacterial products is based on the fact that for over two hundred years neoplasms have been observed to regress following

35 acute infections, principally streptococcal. If these cases were not too far advanced and the infections were

of sufficient severity or duration, the tumors completely disappeared and the patients remained free from recurrence.

Shear, 1950, J. A.M.A. 142:383-390 (Shear), observed that 75 percent of the spontaneous remissions in untreated leukemia in the Children's Hospital in Boston occurred following an acute episode of bacterial infection. Shear questioned:

10 Are pathogenic and non-pathogenic organisms one of Nature's controls of microscopic foci of malignant disease, and in making progress in the control of infectious diseases, are we removing one of Nature's controls of cancer?

Subsequent evidence from a number of research laboratories indicated that at least some of the anti-cancer effects are mediated through stimulation of the host immune system, resulting in enhanced immuno-rejection of the cancer cells. For example, release of the lipopolysaccharide (LPS) endotoxin by gram-negative bacteria such as *Salmonella* 20 triggers release of tumor necrosis factor, TNF, by cells of the host immune system, such as macrophages, Christ et al., 1995, Science 268:80-83. Elevated TNF levels in turn initiate a cascade of cytokine-mediated reactions which culminate in the death of tumor cells. In this regard, 25 Carswell et al., 1975, Proc. Natl. Acad. Sci. USA 72:3666-3669, demonstrated that mice injected with bacillus Calmette-Guerin (BCG) have increased serum levels of TNF and that TNF-positive serum caused necrosis of the sarcoma Meth A and other transplanted tumors in mice. Further, Klimpel et al., 30 1990, J. Immunol. 145:711-717, showed that fibroblasts infected *in vitro* with *Shigella* or *Salmonella* had increased susceptibility to TNF.

As a result of such observations as described above, immunization of cancer patients with BCG injections is 35 currently utilized in some cancer therapy protocols. See Sosnowski, 1994, Compr. Ther. 20:695-701; Barth and Morton, 1995, Cancer 75 (Suppl. 2):726-734; Friberg, 1993, Med.

Oncol. Tumor. Pharmacother. 10:31-36 for reviews of BCG therapy.

2.2 PARASITES AND CANCER CELLS

5 Although the natural biospecificity and evolutionary adaptability of parasites has been recognized for some time and the use of their specialized systems as models for new therapeutic procedures has been suggested, there are few reports of, or proposals for, the actual use of
10 parasites as vectors.

 Lee et al., 1992, Proc. Natl. Acad. Sci. USA 89:1847-1851 (Lee et al.) and Jones et al., 1992, Infect. Immun. 60:2475-2480 (Jones et al.) isolated mutants of *Salmonella typhimurium* that were able to invade HEP-2 (human
15 epidermoid carcinoma) cells *in vitro* in significantly greater numbers than the wild type strain. The "hyperinvasive" mutants were isolated under conditions of aerobic growth of the bacteria that normally repress the ability of wild type strains to invade HEP-2 animal cells. However, Lee et al.
20 and Jones et al. did not suggest the use of such mutants as therapeutic vectors, nor did they suggest the isolation of tumor-specific bacteria by selecting for mutants that show infection preference for melanoma or other cancers over normal cells of the body. Without tumor-specificity or other
25 forms of attenuation, such hyperinvasive *Salmonella typhimurium* as described by Lee et al. and Jones et al. would likely be pan-invasive, causing wide-spread infection in the cancer patient.

30 2.3 TUMOR-TARGETED BACTERIA

 Genetically engineered *Salmonella* have been demonstrated to be capable of tumor targeting, possess anti-tumor activity and are useful in delivering effector genes such as the herpes simplex thymidine kinase (HSV TK) to solid
35 tumors (Pawelek et al., WO 96/40238). Two significant considerations for the *in vivo* use of bacteria are their virulence and ability to induce tumor necrosis factor α

(TNF α)-mediated septic shock. As TNF α -mediated septic shock is among the primary concerns associated with bacteria, modifications which reduce this form of an immune response would be useful because TNF α levels would not become toxic, and a more effective concentration and/or duration of the therapeutic vector could be used.

2.4 MODIFIED BACTERIAL LIPID A

Modifications to the lipid composition of tumor-targeted bacteria which alter the immune response as a result of decreased induction of TNF α production were suggested by Pawelek et al. (Pawelek et al., WO 96/40238). Pawelek et al. provided methods for isolation of genes from *Rhodobacter* responsible for monophosphoryl lipid A (MLA) production. MLA acts as an antagonist to septic shock. Pawelek et al. also suggested the use of genetic modifications in the lipid A biosynthetic pathway, including the mutation *firA*, which codes for the third enzyme UDP-3-O (R-30 hydroxylmyristoyl)-glucosamine N-acyltransferase in lipid A biosynthesis (Kelley et al., 1993, J. Biol. Chem. 268: 19866-19874). Pawelek et al. showed that mutations in the *firA* gene induce lower levels of TNF α . However, these authors did not suggest enzymes which modify the myristate portion of the lipid A molecule. Furthermore, Pawelek et al. did not suggest that modifications to the lipid content of bacteria would alter their sensitivity to certain agents, such as chelating agents.

In *Escherichia coli*, the gene *msbB* (*mlt*) which is responsible for the terminal myristalization of lipid A has been identified (Engel, et al., 1992, J. Bacteriol. 174:6394-6403; Karow and Georgopoulos 1992, J. Bacteriol. 174: 702-710; Somerville et al., 1996, J. Clin. Invest. 97: 359-365). Genetic disruption of this gene results in a stable non-conditional mutation which lowers TNF α induction (Somerville et al., 1996, J. Clin. Invest. 97: 359-365). These references, however, do not suggest that disruption of the *msbB* gene in tumor-targeted *Salmonella* vectors would result

in bacteria which are less virulent and more sensitive to chelating agents.

The problems associated with the use of bacteria as gene delivery vectors center on the general ability of bacteria to directly kill normal mammalian cells as well as their ability to overstimulate the immune system via TNF α which can have toxic consequences for the host (Bone, 1992 JAMA 268: 3452-3455; Dinarello et al., 1993 JAMA 269: 1829-1835). In addition to these factors, resistance to antibiotics can severely complicate coping with the presence of bacteria within the human body (Tschaape, 1996, D T W Dtsch Tierarztl Wochenschr 1996 103:273-7; Ramos et al., 1996, Enferm Infec. Microbiol. Clin. 14: 345-51).

Hone and Powell, WO97/18837 ("Hone and Powell"), disclose methods to produce gram-negative bacteria having non-pyrogenic Lipid A or LPS. Although Hone and Powell broadly asserts that conditional mutations in a large number of genes including *msbB*, *kdsA*, *kdsB*, *kdtA*, and *htrB*, etc. can be introduced into a broad variety of gram-negative bacteria including *E. coli*, *Shigella* sp., *Salmonella* sp., etc., the only mutation exemplified is an *htrB* mutation introduced into *E. coli*. Further, although Hone and Powell propose the therapeutic use of non-pyrogenic *Salmonella* with a mutation in the *msbB* gene, there is no enabling description of how to accomplish such use. Moreover, Hone and Powell propose using non-pyrogenic bacteria only for vaccine purposes.

The objective of a vaccine vector is significantly different from the presently claimed tumor-targeted vectors. Thus, vaccine vectors have requirements quite different from tumor-targeted vectors. Vaccine vectors are intended to elicit an immune response. A preferred live bacterial vaccine must be immunogenic so that it elicits protective immunity; however, the vaccine must not be capable of excessive growth *in vivo* which might result in adverse reactions. According to the teachings of Hone and Powell, a suitable bacterial vaccine vector is temperature sensitive

having minimal replicative ability at normal physiological ranges of body temperature.

In contrast, preferred tumor-targeted parasitic vectors, such as but not limited to *Salmonella*, are safely tolerated by the normal tissues of the body such that pathogenesis is limited, yet the vectors target to tumors and freely replicate within them. Thus, vaccine vectors which replicate minimally at normal body temperatures, would not be suitable for use as tumor-targeted vectors.

- 10 The preferred properties of tumor-specific *Salmonella* strains include 1) serum resistance, allowing the parasite to pass through the vasculature and lymphatic system in the process of seeking tumors, 2) facultative anaerobiasis, i.e., ability to grow under anaerobic or
- 15 aerobic conditions allowing amplification in large necrotic tumors which are hypoxic as well as small metastatic tumors which may be more aerobic, 3) susceptibility to the host's defensive capabilities, limiting replication in normal tissues but not within tumors where the host defensive
- 20 capabilities may be impaired, 4) attenuation of virulence, whereby susceptibility to the host defenses may be increased, and the parasite is tolerated by the host, but does not limit intratumoral replication, 5) invasive capacity towards tumor cells, aiding in tumor targeting and anti-tumor activity, 6)
- 25 motility, aiding in permeation throughout the tumor, 7) antibiotic sensitivity for control during treatment and for post treatment elimination (e.g., sensitivity to ampicillin, chloramphenicol, gentamicin, ciprofloxacin), and lacking antibiotic resistance markers such as those used in strain
- 30 construction, and 8) low reversion rates of phenotypes aiding in the safety to the recipient individual.

3. SUMMARY OF THE INVENTION

The present invention provides a means to enhance the safety of tumor-targeted bacteria, for example, by genetic modification of the lipid A molecule. The modified tumor-targeted bacteria of the present invention induce TNF α

less than the wild type bacteria and have reduced ability to directly kill normal mammalian cells or cause systemic disease compared to the wild type strain. The modified tumor-targeted bacteria of the present invention have
5 increased therapeutic efficacy, i.e., more effective dosages of bacteria can be used and for extended time periods due to the lower toxicity in the form of less induced TNF α and systemic disease.

The present invention provides compositions and
10 methods for the genetic disruption of the *msbB* gene in bacteria, such as *Salmonella*, which results in bacteria, such as *Salmonella*, possessing a lesser ability to elicit TNF α and reduced virulence compared to the wild type. In one embodiment, the invention provides for improved methods for
15 selecting genetic disruptions of the *msbB* gene. Additionally, the genetically modified bacteria have increased sensitivity to a chelating agent compared to bacteria with the wild type *msbB* gene. In a preferred embodiment, *Salmonella* having a disrupted *msbB* gene, which
20 are hyperinvasive to tumor tissues, are able to replicate within the tumors, and are useful for inhibiting the growth and/or reducing the tumor volume of sarcomas, carcinomas, lymphomas or other solid tumor cancers, such as germ line tumors and tumors of the central nervous system, including,
25 but not limited to, breast cancer, prostate cancer, cervical cancer, uterine cancer, lung cancer, ovarian cancer, testicular cancer, thyroid cancer, astrocytoma, glioma, pancreatic cancer, stomach cancer, liver cancer, colon cancer, and melanoma.

30 In an embodiment of the present invention, the bacteria are attenuated by other means, including but not limited biosynthetic pathway mutations leading to auxotrophy. In one specific embodiment, the biosynthetic pathway mutation is a genetic disruption of the *purI* gene. In another
35 embodiment, the bacteria express pro-drug converting enzymes including but not limited to HSV-TK, cytosine deaminase (CD), and p450 oxidoreductase.

The present invention also provides a means for enhanced sensitivity for use in terminating therapy and for post therapy elimination. According to one embodiment of the present invention, the tumor-targeted bacteria having a
5 genetically modified lipid A also have enhanced susceptibility to certain agents, e.g., chelating agents. It is a further advantage to modify tumor-targeted bacteria in this way because it increases the ability to eliminate the bacteria with agents which have an antibiotic-like effect,
10 such as chelating agents including, but not limited to, Ethylenediaminetetraacetic Acid (EDTA), Ethylene Glycol-bis(β -aminoethyl Ether) N, N, N', N',-Tetraacetic Acid (EGTA), and sodium citrate. Modification to enhance the ability to eliminate the bacteria via exogenous means, such
15 as the administration of an agent to which the genetically modified bacteria are more sensitive than their wild type counterparts, is therefore useful.

The present invention further provides for a *Salmonella* strain comprising deletion mutations in both the
20 *msbB* gene as well as an auxotrophic gene. In a specific embodiment, the auxotrophic deletion mutation affects the *purI* gene. In a preferred embodiment, these mutations lead to increased safety of the strain. In another preferred embodiment, the strain also carries other mutations described
25 herein which increase efficacy of the strain but are not essential for its safety.

4. DEFINITIONS

As used herein, *Salmonella* encompasses all
30 *Salmonella* species, including: *Salmonella typhi*, *Salmonella choleraesuis*, and *Salmonella enteritidis*. Serotypes of *Salmonella* are also encompassed herein, for example, *typhimurium*, a subgroup of *Salmonella enteritidis*, commonly referred to as *Salmonella typhimurium*.

35 Attenuation: Attenuation is a modification so that a microorganism or vector is less pathogenic. The end result of attenuation is that the risk of toxicity as well as other

side-effects is decreased, when the microorganism or vector is administered to the patient.

Virulence: Virulence is a relative term describing the general ability to cause disease, including the ability to kill normal cells or the ability to elicit septic shock (see specific definition below).

Septic shock: Septic shock is a state of internal organ failure due to a complex cytokine cascade, initiated by $\text{TNF}\alpha$. The relative ability of a microorganism or vector to elicit $\text{TNF}\alpha$ is used as one measure to indicate its relative ability to induce septic shock.

Chelating agent sensitivity: Chelating agent sensitivity is defined as the effective concentration at which bacteria proliferation is affected, or the concentration at which the viability of bacteria, as determined by recoverable colony forming units (c.f.u.), is reduced.

5. BRIEF DESCRIPTION OF THE FIGURES

The present invention may be understood more fully by reference to the following detailed description, illustrative examples of specific embodiments and the appended figures.

FIG. 1. The complete DNA sequence of the *Salmonella* wild type (WT) 14028 *msbB* gene (SEQ ID NO:1) and the deduced amino acid sequence of the encoded protein (SEQ ID NO:2).

FIG. 2A-2C. Knockout construct generated using the cloned *Salmonella* WT 14028 *msbB* gene. The cloned gene was cut with *Sph*I and *Mlu*I thereby removing approximately half of the *msbB* coding sequence, and the tetracycline resistance gene (TET) from pBR322 cut with *Aat*II and *Ava*I was inserted after blunt-ending using the Klenow fragment of DNA polymerase I. A = Knockout construct. B = *Salmonella* chromosomal copy of *msbB*. C = *Salmonella* disrupted

chromosomal copy of *msbB* after homologous recombination. The start codon (ATG) and stop codon (TAA) and restriction sites *AseI*, *BamHI*, *SphI*, *MluI*, and *EcoRV* are shown. The position of two primers, P1 and P2 which generate two different sized PCR products for either wild type or disrupted *msbB* are shown.

FIG. 3A-3C. Southern blot analysis of chromosomally disrupted *Salmonella* WT 14028 *msbB*.

- 10 A) Southern blot probed with the tetracycline gene, demonstrating its presence in the plasmid construct and the two clones, and its absence in the WT 14028 bacteria.
- B) Southern blot of a similar gel probed with an ³²P-labeled *AseI*/*BamHI* fragment derived from the cloned *msbB*. The *AseI* enzyme cuts upstream of *msbB*, and the *BamHI* cuts in one location in the wild type, but in a second location in the tetracycline gene which results in a higher molecular weight product. Lane 1 (KO) shows the position of the band in the knockout construct, compared to the WT 14028 in lane 2 (WT).
- 15 20 Lanes 3 and 4 show the clones YS8211 and YS861 with a higher molecular weight product. C) Southern blot of a similar gel probed with an ³²P-labeled *mluI* fragment derived from the cloned *msbB*. See text Section 7.2 for details.

- 25 **FIG. 4.** TNF α induction by live *Salmonella* WT 14028 in mice. 1×10^8 live bacteria in 0.1cc phosphate buffered saline of the wild type or *msbB*⁻ disrupted strains were injected i.v. in the tail vein of Balb/c mice. The bar graph indicates the TNF α induction with error bars. Clone YS8211
- 30 induces TNF α 32% compared to *Salmonella* WT 14028.

FIG. 5. TNF α response by Sinclair swine to live *Salmonella* WT 14028 and *msbB*⁻ clone YS8212. TNF α levels were measured at 1.5 and 6.0 hours following i.v. introduction of

35 1×10^9 c.f.u. *Salmonella* WT 14028 and YS8212. At 1.5 hours TNF α response was significantly lower ($p \leq 0.011$) in the *msbB* deletion mutant compared to the wild type.

FIG. 6A-6B. Respiratory level changes induced by LPS from WT 14028 and *msbB*⁻ clone YS8212. Sinclair swine were injected with A) 5 µg/kg purified LPS or B) 500 µg/kg purified LPS and respiration rate was determined. The 500 µg/kg of LPS from *Salmonella* WT 14028 raised the rate of respiration to more than 4 times normal, whereas the rate of respiration in *msbB*⁻ LPS-treated animals was less than doubled.

10 **FIG. 7.** TNFα induction by live *Salmonella* WT 14028 in human monocytes. Human monocytes isolated from peripheral blood were exposed to increasing amounts of *Salmonella* c.f.u. At 1.0×10^5 c.f.u., concentrations of TNFα induced by WT 14028 were more than 3 times higher than those induced by a
15 number of *msbB*⁻ clones, i.e., YS8211, YS8212, YS8658, and YS1170.

FIG. 8. TNFα production by human monocytes. Human monocytes isolated from peripheral blood were exposed to
20 increasing amounts of purified LPS. As little as 1 nanogram of LPS from wild type was sufficient to elicit a measurable TNFα response and was maximal at 10 ng. In contrast, 100 µg of LPS from each of a number of *msbB*⁻ clones was insufficient to generate any response. Thus, at 10 ng LPS, the
25 concentration of TNFα induced by *Salmonella* WT 14028 was at least 10^5 times higher than concentrations of TNFα induced by the independent *msbB* knockouts, i.e., YS7216 and YS8211, and the derivatives, i.e., YS1170, YS8644, YS1604, YS8212, YS8658, YS1601, YS1629.

30 **FIG. 9A-9B.** Survival of mice and Sinclair swine, injected with 2×10^7 or 1×10^9 respectively of live bacteria. A) WT 14028 killed all the mice in 4 days, whereas the *msbB*⁻ clone YS862 spared 90% of the mice past 20 days. B)
35 Similarly, WT 14028 killed all the swine in 3 days, whereas the *msbB*⁻ clone YS8212 spared 100% of the swine past 20 days.

FIG. 10. Biodistribution of *msbB*⁻ *Salmonella* YS8211 in B16F10 melanoma tumors. At 5 days, the ratio of *msbB*⁻ *Salmonella* within the tumors compared to those in the liver exceeded 1000:1.

5

FIG. 11. Tumor retardation by *msbB*⁻ *Salmonella*. B16F10 melanoma tumors were implanted in the flank of C57BL/6 mice and allowed to progress to day 8. Mice either received no bacteria (control) or *msbB*⁻ strains YS8211, YS8212, YS7216, 10 YS1629. Two of the strains, YS8211 and YS1629 retarded tumor progression significantly, whereas strains YS7216 and YS8212 did not.

FIG. 12A-12B. Sensitivity of WT 14028 and *msbB* 15 disrupted bacteria to chelating agents. Wild type and *msbB* disrupted *Salmonella* clone YS8211 and YS862 were grown in LB broth lacking sodium chloride (LB-zero), in the presence or absence of 1 mM EDTA (FIG. 12A) or in the presence or absence of 10 mM sodium citrate (FIG. 12B). The OD₆₀₀ was determined 20 and plotted as a function of time. The *msbB*⁺ strain showed little inhibition by EDTA or sodium citrate, compared to the *msbB*⁻ strains which showed near complete cessation of growth after 3 hours for EDTA or sodium citrate.

FIG. 13A-13B. Survival of *msbB*⁻ bacteria within 25 murine macrophages. Murine bone marrow-derived macrophages (FIG. 13A) and a murine macrophage cell line, J774, (FIG. 13B) were used as hosts for bacterial internalization and quantified over time. The data are presented as a percentage 30 of initial c.f.u.

FIG. 14. Conversion of *msbB1*(Δ)::tet to tet^S using the positive selection suicide vector pCVD442 carrying a second version of the *msbB*⁻ (*msbB2*(Δ)amp^R sacB⁺).

35

FIG. 15. Schematic diagram of the derivation of strain YS1456 from wild type *Salmonella typhimurium*. See text Section 8.1 for details.

5 FIG. 16. Schematic diagram of the derivation of strain YS1646 from wild type *Salmonella typhimurium*. See text Section 8.2 for details.

FIG. 17. Effect of YS1646 dose on B16-B10 murine
10 melanoma tumor growth.

FIG. 18. Antibiotic suppression of YS1646-induced mortality following lethal infection.

15

6. DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the isolation of a gene of *Salmonella*, i.e., *msbB*, which, when present in its normal form, contributes to TNF α induction, general
20 virulence, survival within macrophages, and insensitivity to certain agents which promote eradication of the bacteria. The present invention is directed to the genetic modification of the gene which results in disrupting the normal function of the product of the gene, and the incorporation of the
25 genetic modification into tumor-targeted bacteria, including *Salmonella*, for therapeutic use. In a preferred embodiment, the bacteria have a genetic modification of the *msbB* gene as well as genetic modification of a gene in a biosynthetic pathway, such as the *purI* gene, resulting in an auxotrophic
30 strain.

In a preferred embodiment, the genetically modified bacteria are used in animals, including humans, for reduction of volume and/or growth inhibition of solid tumors.

In an additional preferred embodiment, bacteria
35 useful for the present invention show preference for attachment to and penetration into certain solid tumor cancer cells or have an enhanced propensity to proliferate in tumor

tissues as compared to normal tissues. These bacteria, include but are not limited to *Salmonella*, having a natural ability to distinguish between cancerous or neoplastic cells tissues and normal cells/tissues.

5 Alternatively, tumor cell-specific bacteria useful for the invention may be selected for and/or improved in tumor targeting ability using the methods described by Pawelek et al., WO 96/40238 incorporated herein by reference. Pawelek et al. describe methods for isolating tumor cell-
10 specific bacteria by cycling a microorganism through pre-selected target cells, preferably solid tumor cells *in vitro*, or through a solid tumor *in vivo*, using one or more cycles of infection.

15 **6.1 ISOLATION/IDENTIFICATION OF A GENE INVOLVED IN VIRULENCE**

 The *E. coli* gene, *msbB*, has been shown to be involved in myristilization of lipid A (Somerville et al., 1996, J. Clin. Invest. 97:359-365.) The chromosomal
20 organization of the *E. coli msbB* gene and the DNA sequence coding for the *msbB* gene have been described (Engel, et al., 1992, J. Bacteriol. 174:6394-6403; Karow and Georgopoulos, 1992, J. Bacteriol. 174: 702-710; Somerville et al., 1996, J. Clin. Invest. 97: 359-365).

25 As shown in the present invention, the *msbB* gene can be isolated from bacterial strains, other than *E. coli*, using low stringency DNA/DNA hybridization techniques known to those skilled in the art. (Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory Press, 1989). For an
30 illustrative example of isolation of a *msbB* gene of bacteria, including but not limited to *Salmonella* spp., see Section 7.1 *infra*. A bacterial DNA library can be probed with a ³²P-labeled *msbB* gene from *E. coli*. Hybridizing clones are determined to be correct if they contain DNA sequences
35 similar to the known *E. coli msbB* gene.

6.1.1 GENETIC ALTERATION OF SALMONELLA msbB

One embodiment of the present invention provides a composition of matter which is a strain of bacteria with a genetic alteration in the *msbB* gene. In a preferred
5 embodiment, the bacteria is *Salmonella* sp. Genetic alteration in the form of disruption or deletion can be accomplished by several means known to those skilled in the art, including homologous recombination using an antibiotic resistance marker. These methods involve disruption of the
10 plasmid-based, cloned *msbB* gene using restriction endonucleases such that part or all of the gene is disrupted or eliminated or such that the normal transcription and translation are interrupted, and an antibiotic resistance
15 marker for phenotypic selection is inserted in the region of that deletion, disruption or other alteration. Linearized DNA is transformed into *Salmonella*, and bacteria bearing the antibiotic resistance are further examined for evidence of genetic alteration. Means for examining genetic alteration
20 include PCR analysis and Southern blotting. For an illustrative example of genetic disruption of a *Salmonella msbB* gene, see Section 7.2.

In another embodiment of the invention, the *msbB*⁻/antibiotic resistance marker can be transduced into a new
25 bacterial strain. An illustrative example is provided in Section 7.2. Bacteriophage P22 and a *Salmonella msbB*⁻ clone can be grown in zero salt Luria broth and the new phages in the supernate can be used to infect a new *Salmonella* strain.

Yet another embodiment of the present invention
30 provides *Salmonella* that are attenuated in more than one manner, e.g., a mutation in the pathway for lipid A production, such as the *msbB* mutation described herein and one or more mutations to auxotrophy for one or more nutrients or metabolites, such as uracil biosynthesis, purine
35 biosynthesis, and arginine biosynthesis as described by Bochner, 1980, J. Bacteriol. 143:926-933 herein incorporated by reference. In a preferred embodiment, the ability of *msbB*⁻

Salmonella to accumulate within tumors is retained by *msbB*⁻ *Salmonella* having one or more mutations resulting in an auxotrophic strain. In a more preferred mode of this embodiment of the invention, the bacterial vector which
5 selectively targets tumors and expresses a pro-drug converting enzyme is auxotrophic for uracil, aromatic amino acids, isoleucine and valine and synthesizes an altered lipid A. In a specific preferred embodiment the *msbB*⁻ *Salmonella* also contain a genetic modification of the
10 biosynthetic pathway gene, *purI*, leading to decreased virulence of the strain compared to wild type. An illustrative example is provided in Sections 7 and 8.

6.1.2 CHARACTERISTICS OF *SALMONELLA* HAVING DISRUPTED *msbB*

15 A characteristic of the *msbB*⁻ *Salmonella*, described herein, is decreased ability to induce a TNF α response compared to the wild type bacterial vector. Both the whole bacteria and isolated or purified lipopolysaccharide (LPS)
20 elicit a TNF α response. In an embodiment of the invention, the *msbB*⁻ *Salmonella* induce TNF α expression at about 5 percent to about 40 percent compared to the wild type *Salmonella* sp. (in other words, the *msbB*⁻ *Salmonella* induce TNF α expression at about 5 percent to about 40 percent of the level induced
25 by wild type *Salmonella*, e.g., WT 14028.) In a preferred embodiment of the invention, the *msbB*⁻ *Salmonella* induce TNF α expression at about 10 percent to about 35 percent of that induced by a wild type *Salmonella* sp. In an embodiment of the invention, purified LPS from *msbB*⁻ *Salmonella* induces
30 TNF α expression at a level which is less than or equal to 0.001 percent of the level induced by LPS purified from wild type *Salmonella* sp. TNF α response induced by whole bacteria or isolated or purified LPS can be assessed *in vitro* or *in vivo* using commercially available assay systems such as by
35 enzyme linked immunoassay (ELISA). For illustrative examples, see sections 7.3.1 and 7.3.2 *infra*. Comparison of TNF α production on a per c.f.u. or on a μ g/kg basis, is used

to determine relative activity. Lower TNF α levels on a per unit basis indicate decreased induction of TNF α production.

REDUCTION OF VIRULENCE

5 Another characteristic of the *msbB*⁻ *Salmonella*, described herein, is decreased virulence towards the host cancer patient compared to the wild type bacterial vector. Wild type *Salmonella* can under some circumstances exhibit the ability to cause significant progressive disease. Acute
10 lethality can be determined for normal wild type live *Salmonella* and live *msbB*⁻ *Salmonella* using animal models. For an illustrative example, see Section 7.4 and Section 9, Table III. Comparison of animal survival for a fixed inoculum is used to determine relative virulence. Strains having a
15 higher rate of survival have decreased virulence.

DECREASED SURVIVAL WITHIN MACROPHAGES

Another characteristic of *msbB*⁻ *Salmonella* described herein, is decreased survival within macrophage cells as
20 compared to survival of wild type bacteria. Wild type *Salmonella* (e.g., ATCC 14028) are noted for their ability to survive within macrophages (Baumler, et al., 1994, Infect. Immun. 62:1623-1630; Buchmeier and Heffron 1989, Infect. Immun. 57:1-7; Buchmeier and Heffron, 1990, Science 248:730-
25 732; Buchmeier et al., 1993, Mol. Microbiol. 7:933-936; Fields et al., 1986, Proc. Natl. Acad. Sci. USA 83:5189-93; Fields et al., 1989, Science 243:1059-62; Fierer et al., 1993, Infect. Immun. 61:5231-5236; Lindgren et al., 1996, Proc. Natl. Acad. Sci. USA 3197-4201; Miller et al., 1989,
30 Proc. Natl. Acad. Sci. USA 86:5054-5058; Sizemore et al., 1997, Infect. Immun. 65:309-312).

A comparison of survival time in macrophages can be made using an *in vitro* cell culture assay. A lower number of c.f.u. over time is indicative of reduced survival within
35 macrophages. For an illustrative example, see Section 8 *infra*. As shown therein, using the gentamicin-based internalization assay and bone marrow-derived murine

macrophages or the murine macrophage cell line J774, a comparison of survival of WT 14028 and *msbB*⁻ clone YS8211 was determined. In an embodiment of the invention, survival occurs at about 50 percent to about 30 percent; preferably at about 30 percent to about 10 percent; more preferably at about 10 percent to about 1 percent of survival of the wild type stain.

INCREASED SENSITIVITY

10 Another characteristic of one embodiment of the *msbB*⁻ *Salmonella*, described herein, is increased sensitivity of the tumor-targeted bacteria to specific chemical agents which is advantageously useful to assist in the elimination of the bacteria after administration *in vivo*. Bacteria are
15 susceptible to a wide range of antibiotic classes. However, it has surprisingly been discovered that certain *Salmonella msbB*⁻ mutants encompassed by the present invention are sensitive to certain chemicals which are not normally considered antibacterial agents. In particular, certain *msbB*⁻
20 *Salmonella* mutants are more sensitive than WT 14028 to chelating agents.

Previous descriptions of *msbB*⁻ *E. coli* have not suggested increased sensitivity to such chelating agents. To the contrary, reports have included increased resistance to
25 detergents such as deoxycholate (Karow and Georgopoulos 1992 J. Bacteriol. 174: 702-710).

To determine sensitivity to chemical agents, normal wild type bacteria and *msbB*⁻ bacteria are compared for growth in the presence or absence of a chelating agent, for example,
30 EDTA, EGTA or sodium citrate. Comparison of growth is measured as a function of optical density, *i.e.*, a lower optical density in the *msbB*⁻ strain grown in the presence of an agent, than when the strain is grown in its absence, indicates sensitivity. Furthermore, a lower optical density
35 in the *msbB*⁻ strain grown in the presence of an agent, compared to the *msbB*⁺ strain grown in its presence, indicates sensitivity specifically due to the *msbB* mutation. For an

illustrative example, see section 7.7 *infra*. In an embodiment of the invention, 90 percent inhibition of growth of *msbB*⁻ *Salmonella* (compared to growth of wild type *Salmonella* sp.) occurs at about 0.25 mM EDTA to about 0.5 mM EDTA, preferably at about 99 percent inhibition at about 0.25 mM EDTA to above 0.5 mM EDTA, more preferably at greater than 99 percent inhibition at about 0.25 mM EDTA to about 0.5 mM EDTA. Similar range of growth inhibition is observed at similar concentrations of EGTA.

10

DERIVATIVES OF *msbB* MUTANTS

When grown in Luria Broth (LB) containing zero salt, the *msbB*⁻ mutants of the present invention are stable, i.e., produce few derivatives (as defined below). Continued growth of the *msbB*⁻ mutants on modified LB (10 g tryptone, 5 g yeast extract, 2 ml 1N CaCl₂, and 2 ml 1N MgSO₄ per liter, adjusted to pH 7 using 1N NaOH) also maintains stable mutants.

In contrast, when grown in normal LB, the *msbB*⁻ mutants may give rise to derivatives. As used herein, "derivatives" is intended to mean spontaneous variants of the *msbB*⁻ mutants characterized by a different level of virulence, tumor inhibitory activity and/or sensitivity to a chelating agent when compared to the original *msbB*⁻ mutant. The level of virulence, tumor inhibitory activity, and sensitivity to a chelating agent of a derivative may be greater, equivalent, or less compared to the original *msbB*⁻ mutant.

Derivatives of *msbB*⁻ strains grow faster on unmodified LB than the original *msbB*⁻ strains. In addition, derivatives can be recognized by their ability to grow on MacConkey agar (an agar which contains bile salts) and by their resistance to chelating agents, such as EGTA and EDTA. Derivatives can be stably preserved by cryopreservation at -70°C or lyophilization according to methods well known in the art (Cryz et al., 1990, In New Generation Vaccines, M.M. Levine (ed.), Marcel Dekker, New York pp. 921-932; Adams, 1996, In Methods in Molecular Medicine: Vaccine Protocols,

Robinson et al. (eds), Humana Press, New Jersey, pp. 167-185; Griffiths, *Id.* pp. 269-288.)

Virulence is determined by evaluation of the administered dose at which half of the animals die (LD_{50}).

5 Comparison of the LD_{50} of the derivatives can be used to assess the comparative virulence. Decrease in the LD_{50} of a spontaneous derivative as compared to its *msbB*⁻ parent, indicates an increase in virulence. In an illustrative example, the faster-growing derivatives either exhibit the
10 same level of virulence, a greater level of virulence, or a lower level of virulence compared to their respective original mutant strains (see Section 9, Table III.) In another example, the ability of a derivative to induce $TNF\alpha$ remains the same as the original mutant strain (see Section
15 7.3, FIG. 7).

In an illustrative example, the derivatives can either inhibit tumor growth more than or less than their respective original mutant strains (see Section 7.6, FIG. 11). It is demonstrated in Section 7.6 that the original
20 *msbB*⁻ mutant, YS8211, significantly inhibits tumor growth whereas a derivative of this clone, YS8212, has less tumor growth inhibition activity. In contrast, the derivative, YS1629, exhibits enhanced tumor growth inhibition activity compared to its parent *msbB*⁻ clone, YS7216.

25 A derivative which is more virulent than its parent mutant but which does induce $TNF\alpha$ at a lower level when compared to the wild type, i.e., at a level of about 5 percent to about 40 percent of that induced by the wild type *Salmonella*, can be further modified to contain one or more
30 mutations to auxotrophy. In an illustrative example, the YS1170 derivative is mutated such that it is auxotrophic for one or more aromatic amino acids, e.g., *aroA*, and thus can be made less virulent and is useful according to the methods of the present invention. In an additional illustrative
35 example, genetic modifications of the *purI* gene (involved in purine biosynthesis) yield *Salmonella* strains that are less virulent than the parent strain. (See Sections 7 and 8).

Prior to use of a derivative in the methods of the invention, the derivative is assessed to determine its level of virulence, ability to induce TNF α , ability to inhibit tumor growth, and sensitivity to a chelating agent.

5

**6.2 USE OF SALMONELLA WITH DISRUPTED *msbB* FOR
TUMOR TARGETING AND IN VIVO TREATMENT OF
SOLID TUMORS**

According to the present invention, the *msbB*⁻ mutant *Salmonella* are advantageously used in methods to produce a
10 tumor growth inhibitory response or a reduction of tumor volume in an animal including a human patient having a solid tumor cancer. For such applications, it is advantageous that the *msbB*⁻ mutant *Salmonella* possess tumor targeting ability or target preferably to tumor cells/tissues rather than normal
15 cells/tissues. Additionally, it is advantageous that the *msbB*⁻ mutant *Salmonella* possess the ability to retard or reduce tumor growth and/or deliver a gene or gene product that retards or reduces tumor growth. Tumor targeting ability can be assessed by a variety of methods known to
20 those skilled in the art, including but not limited to cancer animal models.

For example, *Salmonella* with a *msbB*⁻ modification are assayed to determine if they possess tumor targeting ability using the B16F10 melanoma subcutaneous animal model.
25 A positive ratio of tumor to liver indicates that the genetically modified *Salmonella* possesses tumor targeting ability. For an illustrative example, see Section 7.5.

Salmonella with the *msbB*⁻ modification can be assayed to determine if they possess anti-tumor ability using
30 any of a number of standard *in vivo* models, for example, the B16F10 melanoma subcutaneous animal model. By way of an illustrative example, and not by way of limitation, tumors are implanted in the flanks of mice and staged to day 8 and then bacterial strains are injected i.p.. Tumor volume is
35 monitored over time. Anti-tumor activity is determined to be present if tumors are smaller in the bacteria-containing

groups than in the untreated tumor-containing animals. For an illustrative example, see section 7.6 *infra*.

The *Salmonella* of the present invention for *in vivo* treatment are genetically modified such that, when
5 administered to a host, the bacteria is less toxic to the host and easier to eradicate from the host's system. The *Salmonella* are super-infective, attenuated and specific for a target tumor cell. In a more preferred embodiment, the *Salmonella* may be sensitive to chelating agents having
10 antibiotic-like activity.

In addition, the *Salmonella* used in the methods of the invention can encode "suicide genes", such as pro-drug converting enzymes or other genes, which are expressed and secreted by the *Salmonella* in or near the target tumor.
15 Table 2 of Pawelek et al. WO96/40238 at pages 34-35 presents an illustrative list of pro-drug converting enzymes which are usefully secreted or expressed by *msbB*⁻ mutant *Salmonella* for use in the methods of the invention. Table 2 and pages 32-35 are incorporated herein by reference. The gene can be under
20 the control of either constitutive, inducible or cell-type specific promoters. See Pawelek et al. at pages 35-43, incorporated herein by reference, for additional promoters, etc. useful for mutant *Salmonella* for the methods of the present invention. In a preferred embodiment, a suicide gene
25 is expressed and secreted only when a *Salmonella* has invaded the cytoplasm of the target tumor cell, thereby limiting the effects due to expression of the suicide gene to the target site of the tumor.

In a preferred embodiment, the *Salmonella*,
30 administered to the host, expresses the HSV TK gene. Upon concurrent expression of the TK gene and administration of ganciclovir to the host, the ganciclovir is phosphorylated in the periplasm of the microorganism which is freely permeable to nucleotide triphosphates. The phosphorylated ganciclovir,
35 a toxic false DNA precursor, readily passes out of the periplasm of the microorganism and into the cytoplasm and

nucleus of the host cell where it incorporates into host cell DNA, thereby causing the death of the host cell.

The method of the invention for inhibiting growth or reducing volume of a solid tumor comprises administering 5 to a patient having a solid tumor, an effective amount of an isolated mutant *Salmonella* sp. comprising a genetically modified *msbB* gene, said mutant being capable of targeting to the solid tumor when administered *in vivo*. The *msbB* mutant *Salmonella* may also express a suicide gene as described 10 above.

In addition, in one embodiment the isolated *Salmonella* is analyzed for sensitivity to chelating agents to insure for ease in eradication of the *Salmonella* from the patient's body after successful treatment or if the patient 15 experiences complications due to the administration of the isolated *Salmonella*. Thus, if *Salmonella* is employed which is sensitive to a chelating agent, at about 0.25 mM to about 1.0 mM of a chelating agent such as EGTA, EDTA or sodium citrate can be administered to assist in eradication of the 20 *Salmonella* after the anti-tumor effects have been achieved.

When administered to a patient, e.g., an animal for veterinary use or to a human for clinical use, the mutant *Salmonella* can be used alone or may be combined with any physiological carrier such as water, an aqueous solution, 25 normal saline, or other physiologically acceptable excipient. In general, the dosage ranges from about 1.0 c.f.u./kg to about 1×10^{10} c.f.u./kg; optionally from about 1.0 c.f.u./kg to about 1×10^8 c.f.u./kg; optionally from about 1×10^2 c.f.u./kg to about 1×10^8 c.f.u./kg; optionally from about 30 1×10^4 c.f.u./kg to about 1×10^8 c.f.u./kg.

The mutant *Salmonella* of the present invention can be administered by a number of routes, including but not limited to: orally, topically, injection including, but limited to intravenously, intraperitoneally, subcutaneously, 35 intramuscularly, intratumorally, i.e., direct injection into the tumor, etc.

The following series of examples are presented by way of illustration and not by way of limitation on the scope of the invention.

5 7. **EXAMPLE: LOSS OF VIRULENCE, REDUCED TNF α STIMULATION, AND INCREASED CHELATING AGENT SENSITIVITY, BY DISRUPTION OF THE SALMONELLA msbB**

7.1 ISOLATION AND COMPOSITION OF SALMONELLA msbB GENE

10 A *Salmonella* genomic DNA library was first constructed. Wild type *Salmonella* typhimurium (ATCC strain 14028) were grown overnight and genomic DNA extracted according to the methods of Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Press, Cold Spring Harbor, 1989). Size-selected restriction endonuclease-digested fragments ranging from 2 to 10 kb were generated by time-limited digestion with Sau3A and selected by agarose gel electrophoresis. These fragments were ligated into pBluescript SK- and transformed to *E. coli* DH5 α . Random
15 analysis of clones revealed DNA inserts in $\geq 87\%$, with average size = 5.1 Kb. The library consisted of 1.4×10^4 independent clones. In order to reduce the hybridization of the *E. coli*-originated *msbB* probe, to the 100% homologous chromosomal gene in *E. coli*, the entire library was harvested
20 from the petri dishes by flooding them with phosphate buffered saline and using a glass rod to dislodge the colonies, and the resulting bacterial population was subjected to a large-scale plasmid isolation, resulting in an amplified *Salmonella* library plasmid pool. This plasmid pool
25 was then transformed to *Salmonella* LT2 YS5010, thereby eliminating the *E. coli* background.

 A probe for *msbB* homologues was generated using a clone of the *E. coli* *msbB* gene (Karow and Georgopoulos 1992 J. Bacteriol. 174: 702-710) by digesting *E. coli* with
35 *Bgl*III/*Hinc*II and isolating a 600 bp fragment which corresponds to a portion of the coding sequence. This

fragment was labeled using $\alpha^{32}\text{P}$ -dCTP and used to probe the *Salmonella* library at low-stringency conditions consisting of 6X SSC, 0.1 % SDS, 2X Denhardts, 0.5 % non-fat dry milk overnight at 55° C. Strongly hybridizing colonies were
5 purified, and plasmids extracted and subjected to restriction digestion and *in situ* gel hybridization under the same conditions used for colony hybridization (Ehtesham and Hasnain 1991 BioTechniques 11: 718-721). Further restriction digests revealed a 1.5 kb fragment of DNA which strongly
10 hybridized with the probe and was sequenced at the Yale University Boyer Center using fluorescent dye termination thermal cycle sequencing. Sequence analysis revealed that the 1.5 kb fragment contained an *msbB* homologue which apparently lacked an initiating methionine corresponding to
15 that of the *E. coli* gene. A probe consisting of the 5' region of this clone was generated by performing restriction digests using *EcoRI*/*XbaI* and again hybridizing to the library. The complete nucleotide sequence of the *Salmonella msbB* gene (SEQ ID NO:1) and the deduced amino acid sequence
20 of the encoded protein (SEQ ID NO:2) is shown in FIG. 1. The DNA homology of the putative *Salmonella msbB* and the *E. coli msbB* is 75%. The protein homology is 98%, confirming that the cloned *Salmonella* gene is a bona fide *msbB*.

25 7.2 GENETIC ALTERATION OF *SALMONELLA msbB*

A knockout construct was generated using the cloned *Salmonella msbB* gene. The cloned gene was cut with *SphI* and *MluI*, thereby removing approximately half of the *msbB* coding sequence, and the tetracycline resistance gene from pBR322,
30 cut with *AatII* and *AvaI*, was inserted after blunt-ending using the Klenow fragment of DNA polymerase I (FIG. 2A-2C). The knockout disruption was accomplished by homologous recombination procedures (Russell et al., 1989, J. Bacteriol. 171:2609); the construct was linearized using *SacI* and *KpnI*,
35 gel purified and transfected to *Salmonella* LT2 YS501 by electroporation. Bacteria from the transformation protocol were first selected on tetracycline plates, and subsequently

examined for the presence of plasmid-containing non-chromosomal integrated contaminants by ampicillin resistance and the presence of plasmids as determined by standard plasmid mini-preps (Titus, D. E., ed. *Promega Protocols and Applications Guide*, Promega Corp, 1991). Bacterial colonies which were tetracycline resistant yet lacked plasmids were subjected to a PCR-based analysis of the structure of their *msbB* gene. PCR was used with primers which generate a fragment inclusive of the region into which the tetracycline gene was inserted, where the forward primer was GTTGACTGGGAAGGTCTGGAG (SEQ ID NO:3), corresponding to bases 586 to 606, and the reverse primer was CTGACCGCGCTCTATCGCGG (SEQ ID NO:4), corresponding to bases 1465 to 1485. Wild type *Salmonella msbB*⁺ results in an approximately 900 base pair product, whereas the disrupted gene with the tetracycline insert results in an approximately 1850 base pair product. Several clones were obtained where only the larger PCR product was produced, indicating that the disruption in the *msbB* gene had occurred.

20 Southern blot analysis was used to confirm the disruption of the chromosomal copy of *Salmonella msbB*. The plasmid-based knockout construct (KO) was compared with genomic DNA prepared from wild type and putative disrupted *msbB* clones, YS82, YS86, YS8211 and YS861. The DNA was double digested with *AseI*/*Bam*HI and separated by agarose gel electrophoresis on 0.9% or 1.2% agarose. Results of YS8211 and YS861 are presented in FIG. 3A-3C. Similar gels were subjected to three separate criteria: 3A) the presence of the tetracycline gene when probed with an ³²P-labeled tetracycline gene fragment, 3B) Restriction fragment length when probed with an ³²P-labeled *AseI*/*Bam*HI fragment derived from the cloned *msbB* and 3C) the presence or absence of the *msbB* *mluI* fragment removed in order to disrupt the *msbB* gene and insert the tetracycline gene (FIG. 3A-3C). Since the *mluI* fragment was removed in order to disrupt the *msbB* gene and insert the tetracycline gene, it is expected that this probe would hybridize with the wild type FIG. 3C (lane 2 WT) but not the

knockout construct (lane 1 KO), or the clones, (lanes 3 and 4 YS8211 and YS821) thereby confirming the genetic alteration of the *msbB* gene. Each of the clones examined exhibited all of the expected criteria for an *msbB* gene deletion

5 (knockout). These data further confirm that *msbB* exists as a single copy in the wild type *Salmonella*, as no other hybridizing bands were observed when probed with a labeled oligonucleotide derived from the cloned DNA.

After the *msbB* mutation was confirmed, additional
10 strains containing the *msbB*⁻ mutation were generated. The *Salmonella* strains used included WT 14028 and YS72 (*pur*⁻ *xyl*⁻ hyperinvasive mutant from WT 14028; Pawelek et al., WO 96/40238). P22 transduction was used to generate YS8211 (*msbB*::*tet*) using YS82 as a donor and YS861 and YS862
15 (*msbB1*::*tet*) using YS86 as a donor; all with WT 14028 as recipient. YS7216 (*msbB1*::*tet* from YS72) was generated by transduction using YS82 as a donor. Several derivatives are encompassed by the present invention, including but not limited to derivatives of YS8211 (YS8212, YS1170), YS862
20 (YS8644, YS8658), and YS7216 (YS1601, YS1604, YS1629). In a preferred embodiment, spontaneous derivatives grow somewhat faster on Luria agar compared to WT 14028 or *msbB*⁻ clones generated by transduction. *msbB*⁺ strains were grown in LB broth or on LB plates containing 1.5% agar at 37°C. *msbB*⁻
25 strains were grown in modified LB containing 10 g tryptone, 5 g yeast extract, 2 ml 1N CaCl₂ and 2 ml 1N MgSO₄ per liter, adjusted to pH 7 using 1N NaOH. For transducing *msbB1*::*tet*, LB lacking NaCl was used, with 4 mg/l tetracycline. Liquid cultures were shaken at 225 rpm. For tumor targeting
30 experiments, cells were diluted 1:100 in LB, grown to OD₆₀₀=0.8 to 1.0, washed in phosphate buffered saline (PBS), and resuspended in PBS.

7.2.1 AN IMPROVED METHOD FOR SELECTING *msbB*
GENETIC ALTERATIONS BY PRE-SELECTION
WITH SUCROSE

An improved method for selecting *msbB* genetic alterations by pre-selection with sucrose has been discovered. This pre-selection method is based on the selection of colonies that retain the *sacB* gene. The *sacB* gene is responsible for the conversion of sucrose into a toxic chemical, levan, that is lethal to the host cells, and can therefore be used to select for recombinants. Only those strains that undergo deletion of the *sacB* gene survive on medium containing sucrose and therefore have the sucrose resistance property *suc*^r. As described below, pre-selecting of colonies that retain the *sacB* gene, eliminated the need for dilutions and comparison of sucrose⁽⁺⁾ vs. sucrose⁽⁻⁾ colonies as performed in the normal sucrose selection.

The normal selection procedure for the sucrase system:

E. coli SM10 λ pir carrying a plasmid with the *msbB*(Δ) *bla* and *sacB* genes was used as a donor. The *bla* gene for betalactamase confers resistance to ampicillin. In the normal selection procedure, the donor strain was mated using standard mating procedures, with a *Salmonella* strain into which the plasmid with *msbB*(Δ) *bla* *sacB* was to be introduced. Since the *Salmonella* strain contained a second antibiotic resistance marker (e.g., streptomycin resistance), the recombinant *Salmonella* clones were then selected for dual resistance to ampicillin and streptomycin. To test for resolution of an individual clone, dilutions of each clone were plated on LB lacking sucrose, or LB containing 5% sucrose. Only those strains that underwent deletion or alteration of the *sacB* gene survive on sucrose. Comparison of the number of clones on sucrose⁽⁺⁾ or sucrose⁽⁻⁾ plates, indicates the fraction of bacterial cells that underwent resolution. Sucrose resistant colonies were then further tested for sensitivity to ampicillin and tetracycline. Tet^s and amp^s indicated excision of the *sacB* and *bla* genes during cross-over with the partial *msbB* gene region. PCR was then

used to confirm the *msbB* isoform present in the *tet^s amp^s* clones.

Pre-Selection Protocol for the sucrase system:

5 A variation in the normal sucrase protocol allowed for the screening of increased numbers of colonies, by pre-selecting colonies that retain the *sacB* gene. This pre-selection method eliminated the need for examination and comparison of sucrose⁽⁺⁾ vs. sucrose⁽⁻⁾ from a large number of
10 colonies. After the conjugation procedure described above, the colonies (impure at this stage) were gridded directly to LB plates containing 5% sucrose and grown at 30°C. The resulting impure colonies, which continued to grow, gave rise to survivors on sucrose. Of the sucrose resistant colonies,
15 those which displayed a phenotypic variation of "fuzzy edges" were then subjected to dilution and plated on sucrose (+) or sucrose (-) plates. Colonies were then tested for sensitivity to tetracycline and ampicillin as above, and the *msbB* isoform was confirmed by PCR. This improved method was
20 used to generate strains for P22 phage transduction of *msbB*(Δ) *bla sacB* chromosomal element. These strains were then used to generate the YS1456 and YS1646 stains, which represent preferred embodiments of the novel *msbB* mutations of the present invention (see FIG. 15 and 16).

25

7.3 DISRUPTION OF SALMONELLA *msbB* REDUCES TNF α INDUCTION

7.3.1 TNF α INDUCTION IN MICE

30 WT 14028 and the *msbB*⁻ clone YS8211, were first grown to saturation in LB media at 37° C with shaking at 225 rpm. A 1:100 dilution of these bacterial strains were then transferred to fresh LB and grown to an OD₆₀₀ = 1.0 at 37° C with shaking at 225 rpm. The bacteria were diluted in
35 phosphate buffered saline and 1.0 X 10⁸ c.f.u. (about 5 X 10⁹ c.f.u./kg) were injected into the tail vein of Balb/C mice (n = 4/strain), with PBS as a negative control. After 1.5

hours, serum was harvested in triplicate samples by cardiac puncture, centrifuged to remove the cellular content, and analyzed for TNF α using a Biosource International Cytoscreen ELISA plate, which was read on a Molecular Devices Emax 5 microplate reader.

Results are presented in FIG. 4 and expressed as a percent of the level of TNF α induced by wild type *Salmonella*.

As demonstrated in FIG. 4, YS8211 induced TNF α significantly less than WT 14028. Thus, as shown in FIG. 4, 10 the *msbB*⁻ strain induced TNF α about 33% (i.e., 3 times less) of the wild type *msbB*⁺ strain.

7.3.2 TNF α INDUCTION IN PIGS

An *msbB*⁻ strain of *Salmonella*, YS8212, and WT 14028, 15 were first grown to saturation in LB media at 37° C with shaking at 225 rpm. A 1:100 dilution of these bacterial strains were then transferred to fresh LB and grown to an OD₆₀₀ = 0.8 at 37° C with 225 rpm. The bacteria were washed in phosphate buffered saline and 1.0 X 10⁹ c.f.u. (about 1 X 10⁸ 20 c.f.u./kg) were injected into the ear vein of Sinclair swine (n = 6/strain). After 1.5 and 6.0 hours, serum was harvested, centrifuged to remove the cellular content, and frozen for later analysis. Analysis for TNF α utilized a Genzyme Predicta ELISA plate, which was read using a Gilson 25 spectrophotometer.

Results are presented in FIG. 5 and are expressed as picograms of TNF α /ml serum.

As demonstrated in FIG. 5, at 90 minutes the level of TNF α induced by the *msbB*⁻ strain was significantly lower 30 than that induced by the *Salmonella* WT 14028.

7.3.3 SALMONELLA LPS-INDUCED RESPIRATION IN PIGS

Lipopolysaccharide (LPS) from *Salmonella* WT 14028 35 and the *msbB*⁻ clone, YS8212 was prepared using the procedure described by Galanos et al. (1969 Eur. J. Biochem. 9: 245-249). Briefly, LPS was extracted from bacteria which had

been grown to OD₆₀₀ of 1.0. The bacteria were pelleted by centrifugation, washed twice with distilled water and frozen at -20C. LPS was purified by extraction with a mixture of 18.3 ml H₂O:15 ml phenol in a shaking water bath for 1 hr at 70 C. The mixture was cooled on ice, centrifuged at 20,000 x g for 15 min, and the aqueous phase was removed. LPS was precipitated from the aqueous phase by addition of NaCl to 0.05 M and 2 volumes ethanol and incubation on ice, followed by centrifugation of 2000 x g for 10 min. The precipitation was repeated after redissolving the pellet in 0.05 M NaCl, and the pellet lyophilized. The LPS was dissolved in sterile distilled water, and either 5 µg/kg or 500 µg/kg LPS was injected into the ear vein of Sinclair swine which had been anesthetized with Isoflurane. After 1.5 and 6.0 hours, respiration rate was determined and recorded.

Results are presented in FIG. 6 and are expressed as a percentage of respiration at time zero (t₀).

As demonstrated in FIG. 6, respiration was significantly higher in the pigs administered wild type LPS as compared to those administered the LPS from the *msbB*⁻ strain. Thus, disruption of the *msbB* gene in *Salmonella*, produces a modification in lipid A which results in reduced ability to increase respiration.

7.3.4 TNFα INDUCTION IN HUMAN MONOCYTES

Human monocytes were prepared from peripheral blood by centrifugation through Isolymph (Pharmacia) and allowed to adhere to 24 well plates containing RPMI 1640. *Salmonella* WT 14028 and several of the *msbB*⁻ 14028 strains (YS8211, YS8212, YS8658, and YS1170) were first grown to saturation in LB media at 37° C with shaking at 225 rpm. A 1:100 dilution of these bacterial strains was then transferred to fresh LB and grown to an OD₆₀₀ = 0.8 at 37° C with 225 rpm. The bacteria were added to the cell culture wells and the culture medium was harvested after 2.0 hours, centrifuged to remove the cellular content, and analyzed for TNFα using a Genzyme

Predicta ELISA plate, which was read using a Gilson spectrophotometer.

The data are presented in FIG. 7 and expressed as picograms of TNF α /ml serum.

- 5 As demonstrated in FIG. 7, the *msbB*⁻ strains induced TNF α significantly less than did the wild type strain.

7.3.5 *msbB*- SALMONELLA LPS TNF α INDUCTION IN HUMAN MONOCYTES

- 10 Human monocytes were prepared from peripheral blood by centrifugation through Isolymp (Pharmacia) and allowed to adhere to 24 well plates containing RPMI 1640.

- Lipopolysaccharide (LPS) of wild type and of a number of *msbB*⁻ mutant *Salmonella*, (i.e., YS8211, YS8212, YS8658 and YS1170) was prepared using the procedure described by Galanos et al. 15 (1969 Eur. J. Biochem. 9: 245-249) (see Section 7.3.3 for a brief description). The LPS was dissolved in sterile distilled water, and quantities ranging from 0.001 to 100 ng/ml LPS were added to the cell culture wells. After 15 20 hours the culture medium was harvested, centrifuged to remove the cellular content, and analyzed for TNF α using a Genzyme Predicta ELISA plate, which was read using a Gilson spectrophotometer.

- The data are presented in FIG. 8 and are expressed 25 as picograms of TNF α /ml serum.

As demonstrated in FIG. 8, LPS purified from the *msbB*⁻ strains induced TNF α significantly less than did the LPS from the wild type strain.

30 7.4 DISRUPTION OF SALMONELLA *msbB* REDUCES VIRULENCE

7.4.1 IN MICE

- A culture of wild type *Salmonella* 14028 and one of its *msbB*⁻ *Salmonella* clones, YS862, were grown in LB medium lacking sodium chloride at 37°C with shaking at 250 rpm until 35 the cultures reached an OD₆₀₀ of 0.8. The bacteria were diluted into phosphate buffered saline (PBS) at a ratio of

1:10 and the equivalent of 2×10^7 c.f.u. were injected i.p. into C57BL/6 mice bearing B16F10 melanomas. Survival was determined daily, or at two to four day intervals.

Results are presented in FIG. 9A and are expressed as percent survival.

As shown in FIG. 9A, WT 14028 killed all the mice in 4 days, whereas the *msbB*⁻ mutant spared 90% of the mice past 20 days, demonstrating a significant reduction in virulence by the *msbB*⁻ mutant.

10

7.4.2 IN PIGS

A culture of WT 14028 and one of its *msbB*⁻ *Salmonella* clones, YS8212, were grown in LB medium lacking sodium chloride at 37°C with shaking of 250 RPM until the cultures reached an OD₆₀₀ of 0.8. The bacteria were washed in phosphate buffered saline and 1.0×10^9 were injected into the ear vein of Sinclair swine (n = 4/strain). Survival was determined daily, or at two to four day intervals.

Results are presented in FIG. 9B and are expressed as percent survival.

As shown in FIG. 9B, WT 14028 killed all the swine in 3 days, whereas the *msbB*⁻ mutant spared 100% of the mice past 20 days, demonstrating a significant reduction in virulence.

25

7.5 TUMOR TARGETING OF *msbB*⁻ CLONES

Salmonella WT 14028 with the *msbB*⁻ modification, were assayed to determine if they possessed tumor targeting ability using the B16F10 melanoma subcutaneous animal model. The *msbB*⁻ clone, YS8211, was grown in LB media lacking sodium chloride at 37°C with shaking at 250 rpm to an OD₆₀₀ of 0.8. An aliquot of 2.0×10^6 c.f.u. was injected i.v. into C57BL/6 mice which had been implanted with 2×10^5 B16 melanoma cells 16 days prior to the bacterial infection. At two days and five days post bacterial infection, mice were sacrificed and tumors and livers assayed for the presence of the bacteria by homogenization and plating of serial dilutions.

Results are presented in FIG. 10 and are expressed as c.f.u. bacteria/g tissue. As demonstrated in FIG. 10, a positive ratio of tumor to liver (700:1) was found at 2 days, and increased to a positive ratio of 2000:1 at 5 days. Thus, the *msbB*⁻ mutant maintained the ability to target to a solid cancer tumor.

7.6 USE OF SALMONELLA WITH DISRUPTED *msbB* FOR ANTI-TUMOR ACTIVITY IN VIVO

10 *Salmonella typhimurium* 14028 *msbB*⁻ clones YS8211, YS8212, YS7216, and YS1629 and WT 14028 (control) were grown in LB media lacking sodium chloride at 37°C with shaking at 250 rpm to an OD₆₀₀ of 0.8. An aliquot of 2.0 x 10⁶ c.f.u. was injected i.p. into C57BL/6 mice which had been implanted with 2 x 10⁵ B16 melanoma cells 8 days prior to the bacterial 15 infection. Tumor volume was monitored over time.

Results are presented in FIG. 11. Two of the strains, YS8211 and YS1629, showed significant tumor retardation, i.e., tumor growth inhibition.

20

7.7 INCREASED SENSITIVITY TO CHELATING AGENTS

In order to assess the sensitivity of bacterial strains to chelating agents, bacteria with or without the *msbB* mutation were grown in the presence or absence of 1 mM EDTA or 10 mM sodium citrate in Luria Broth (LB) lacking 25 sodium chloride. An overnight culture of each of the bacterial strains was diluted 1 to 100 in fresh media, and grown at 37°C with shaking at 250 rpm. The effect on growth was determined by spectrophotometric readings at an OD₆₀₀.

30 WT 14028 and *msbB*⁻ clone YS8211 were grown in the presence or absence of 1 mM EDTA (FIG. 12A). EDTA did not inhibit the growth of WT 14028. In contrast, the *msbB*⁻ clone showed near complete cessation of growth after 3 hours in the presence of EDTA.

35 WT 14028 and *msbB*⁻ clone YS862 were grown in the presence and absence of 10 mM sodium citrate (FIG. 12B). The *msbB*⁺ WT 14028 strain showed little inhibition by sodium

citrate compared to the *msbB*⁻ strain which showed near complete cessation of growth after 3 hours in the presence of sodium citrate.

Thus, the *msbB*⁻ *Salmonella* mutants exhibited sensitivity to chelating agents which promote eradication of the bacteria, a characteristic which is similar to an antibiotic effect. It is envisioned that such a characteristic would be advantageous for use of *msbB*⁻ *Salmonella* mutants for *in vivo* therapy.

In order to further assess the sensitivity of *Salmonella* strains to chelating agents, the hyperinvasive *pur* strain YS72, its *msbB*⁻ strain, YS7216, and a derivative of YS7216, YS1629, were grown in the presence of increasing concentrations of EDTA. A fresh culture of YS72, its *msbB*⁻ strain YS7216 and its faster-growing derivative YS1629 were diluted 1 to 100 in fresh, zero salt LB media containing 0, 0.25, 0.5, 1.0 or 2.0 mM EDTA and grown at 37°C with 225 RPM for 4 hours, and c.f.u. was determined by plating serial dilutions onto LB plates (Table I). Greater than 99% inhibition was achieved for the *msbB*⁻ strain YS7216 at concentrations of EDTA greater than 0.25 mM and its derivative YS1629 was inhibited greater than 90% at 0.5 mM and greater than 99% at 2.0 mM. In contrast, although the YS72 clone exhibited some sensitivity to EDTA it was not inhibited at the 90% level even at 2.0 mM.

Table I.

Strain	c.f.u. no EDTA	c.f.u. + EDTA {% inhibition}			
		[0.25 mM]	[0.5 mM]	[1.0 mM]	[2.0 mM]
YS72	3.0 x 10 ⁹	2.4 x 10 ⁹ {20%}	1.5 x 10 ⁹ {50%}	7.3 x 10 ⁸ {75%}	4.8 x 10 ⁸ {84%}
YS7216	6.3 x 10 ⁸	2.1 x 10 ⁶ {99.6%}	1.1 x 10 ⁶ {99.8%}	3.2 x 10 ⁶ {99.4%}	4.3 x 10 ⁶ {99.3%}
YS1629	1.3 x 10 ⁹	6.0 x 10 ⁸ {54%}	1.0 x 10 ⁸ {92%}	2.9 x 10 ⁷ {97%}	7.5 x 10 ⁶ {99.4%}

7.8 BACTERIAL SURVIVAL WITHIN MACROPHAGES

In order to determine the sensitivity of *msbB*⁻ *Salmonella* to macrophages, two types of macrophages were used: (A) bone marrow-derived macrophages obtained from the femurs and tibias of C57BL/6 mice, which were allowed to replicate by addition of supernatant from the LADMAC cell line which secretes macrophage colony stimulating factor (Sklar et al., 1985. J. Cell Physiol. 125:403-412) and (B) J774 cells (a murine macrophage cell line) obtained from America Type Culture Collection (ATCC). *Salmonella* strains used were WT 14028 and its *msbB*⁻ derivatives YS8211 and YS1170. Bacteria were grown to late log phase OD₆₀₀=0.8 and 1 x 10⁶ were allowed to infect a confluent layer of mammalian cells within a 24 well dish for 30 min, after which the extracellular bacteria were removed by washing with culture medium and the addition of 50 µg/ml gentamicin (Elsinghorst, 1994, Methods Enzymol. 236:405-420). Bacteria were counted by plating serial dilutions of the cell layer removed using 0.01% deoxycholate, and expressed as the percent initial c.f.u. over time.

The results are presented in FIG. 13 and expressed as percent c.f.u. per time. The *msbB*⁻ strain shows significantly less survival in macrophages.

25

7.9 LD50 OF *msbB* DERIVATIVES

Spontaneous derivatives of *msbB*⁻ strains YS8211 and YS7216 were selected from *in vitro* culture on non-modified LB medium based upon enhanced growth characteristics. These bacterial strains were grown to OD₆₀₀ of 0.8 and c.f.u. ranging from 1 X 10² to 1 X 10⁸ were injected i.v. into the tail vein of C57BL/6 mice. Acute lethality was determined at 3 days, and the LD₅₀ determined as described by Welkos and O'Brien (Methods in Enzymology 235:29-39, 1994). The results are presented in Table II. Thus, although all the *msbB*⁻ strains have a reduced ability to induce TNFα (See Section 7.3.5), the results demonstrate that strain YS1170 is

significantly less attenuated than other *msbB*⁻ strains and therefore not all *msbB*⁻ strains are useful for providing both reduced TNF α induction and reduced virulence.

5

Table II.

	Strain	LD ₅₀
	WT 14028	1 X 10 ³
	YS8211	4 X 10 ⁶
	YS8212	3.9 X 10 ⁷
10	YS1629	1 X 10 ⁷
	YS1170	1 X 10 ⁶

8. *msbB* MUTATION IN COMBINATION WITH A BIOSYNTHETIC PATHWAY MUTATION

15 In order to assess compatibility with auxotrophic mutations, as measured by retention of the ability to target and replicate within tumors, combinations of the *msbB* mutation with auxotrophic mutations were generated. *msbB*⁺ strains were grown in LB broth or LB plates containing 1.5% 20 agar at 37°C. *msbB*⁻ strains were grown in modified LB containing 10 g tryptone, 5 g yeast extract, 2 ml 1N CaCl₂ and 2 ml 1N MgSO₄ per liter, adjusted to pH 7 using 1N NaOH. For transducing *msbB1::tet*, LB lacking NaCl was used, with 4 mg/l tetracycline. Liquid cultures were shaken at 225 rpm. The 25 *msbB1::tet* was transduced to auxotrophic strains to generate YS1604 (*msbB*⁻, *pur*⁻, hyperinvasive), YS7232 (*msbB*⁻, *purI*⁻, hyperinvasive), YS7244 (*msbB*⁻, *purI*⁻, *aroA*⁻ hyperinvasive), YS1482 (*msbB*⁻, *purI*⁻, *purA*⁻). For tumor targeting experiments, cells were diluted 1:100 into LB, grown to 30 OD₆₀₀=0.8 to 1.0, washed in phosphate buffered saline (PBS), resuspended in PBS, and 2 x 10⁶ were injected into the tail vein of C57BL/6 mice. At day 7, tumors were excised, weighed, homogenized, and c.f.u. determined by plating serial dilutions onto modified LB described above.

35 Results are presented in Table III and are expressed as c.f.u. per gram tumor tissue. Some of the strains, YS8211, YS1604, and YS7232 show high levels of

c.f.u. within the tumors, whereas YS7244 and YS1482 are approximately 500 to 5000 times less.

Table III.

5	Strain	genetic marker	c.f.u./gram tumor tissue
	YS8211	<i>msbB</i> ⁻	3 x 10 ⁹
	YS1604	<i>msbB</i> ⁻ , <i>pur</i> ⁻ , hyperinvasive	9 x 10 ⁹
	YS7232	<i>msbB</i> ⁻ , <i>purI</i> ⁻ , hyperinvasive	9 x 10 ⁹
	YS7244	<i>msbB</i> ⁻ , <i>purI</i> ⁻ , <i>aroA</i> ⁻ hyperinvasive	5 x 10 ⁵
10	YS1482	<i>msbB</i> ⁻ , <i>purI</i> ⁻ , <i>purA</i> ⁻	6 x 10 ⁶

8.1 GENERATION OF THE YS1456 STRAIN CONTAINING DELETIONS IN *msbB* AND *purI*

15 The generation of *Salmonella* strain YS1456 from the wild type *Salmonella typhimurium* is outlined in FIG. 15. The wild type *Salmonella typhimurium* was transduced with *purI* 1757::Tn10 which conferred tetracycline-resistance, resulting in strain YS1451.

20 Strain YS1451 was then subjected to a Bochner selection to render the strain tet sensitive and introduce *tet*^s gene and introduce a *purI* deletion (Bochner et al. 1980, J. Bacteriol. 143:926-933), yielding the strain YS1452. Strain YS1452 was *tet*^s and *purI*⁻. Strain 1452 was then
25 transduced with *msbB1*::*tet* via bacteriophage P22, using strain YS8211 (*msbB*::*tet*) as the donor. The resulting strain, YS1453, was initially sensitive to 10 mM ethylene glycol bis((b-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), spontaneously reverted to a EGTA-resistant phenotype.
30 One such revertant, denoted YS1454, was selected by plating YS1453 on EGTA (2mM in Luria agar).

Strain YS1454 was then transduced with the *msbB2*(Δ) *bla sacB* chromosomal element, selecting for ampicillin resistance. This transduction process brought in a second
35 version of the disrupted *msbB* gene, denoted *msbB2*(Δ) as well as the *bla* and *sacB* genes. The *bla* gene is responsible for the transcription of the enzyme β-lactamase, which

metabolizes ampicillin, and was used to select for ampicillin resistant transductants. The *sacB* gene is responsible for the conversion of sucrose into a toxic chemical, levan, that is lethal to the host cells, and was subsequently used to
5 select for recombinants which lose or have mutations in *sacB* (see Section 7.2.1 for improved pre-selection methods with sucrose). The presence of the *bla* and *sacB* genes allowed the selection of the *amp^r* and *suc^s* strain (denoted as strain YS1455), which contained both the *msbB1::tet* and *msbB2(Δ)*
10 genes.

Strain YS1455 was then plated on Luria Bertani (LB) sucrose to select a *suc^r amp^s tet^s* derivative to remove *msbB1::tet* and restore antibiotic sensitivity. The derivative was denoted as strain YS1456.

15 In summary YS1456 has deletion mutations in *purI* and *msbB*. It is also *tet^s amp^s* and *EGTA^r*.

8.2 GENERATION OF THE YS1646 STRAIN CONTAINING DELETIONS IN *msbB* and *purI*

20 The generation of *Salmonella* strain YS1646 from the wild type *Salmonella typhimurium* (wild type strain ATCC 14028) is outlined in FIG. 16. The wild type *Salmonella typhimurium* was mutagenized with nitrosoguanidine and
25 ultraviolet (UV) light and selected for hyperinvasiveness in melanoma cells. The resistant strain, denoted YS72, were confirmed to possess tumor-hyperinvasiveness *pur⁻* and *xyl⁻* properties (Pawelek et al., 1997, Cancer Res 57: 4537-4544).

To replace the chromosomal *purI* gene in strain YS72
30 with a *purI* deletion, strain YS72 was transduced with the *purI* 1757::Tn10 gene, which conferred tetracycline-resistance. The donor for the *purI* 1757::Tn10 gene was *Salmonella* strain TT11 (*purI* 1757::Tn10). The donor strain was originally obtained from the *Salmonella* Genetic Stock
35 Center (Dept. of Biological Science, Univ. Calgary, Calgary, Alberta, Canada T2N 1N4). Transduction was performed using bacteriophage P22 (mutant HT105/1 int-201). The

transductant, denoted YS1641, was isolated following selection on tetracycline.

Strain YS1641 was then subjected to a Bochner selection to remove the *tet* gene and introduce a *purI* gene deletion (Bochner et al., 1980, J. Bacteriol. 143:926-933), yielding strain YS1642. Strain YS1642 was *tet*^s and *purI*⁻. The selection of a *tet*⁻-deleted strain allowed further genetic modification (e.g., *msbB* gene disruption, see next paragraph) using *tet* gene transduction. Strain YS1642 has a tight
10 purine requirement due to *purI*(Δ), and has been shown to revert to *purI*⁺ at a frequency of less than 1 in 10¹⁰ cells.

Strain YS1642 was then transduced with *msbB1::tet* via bacteriophage P22, using strain YS8211 (*msbB::tet*) as the donor. The DNA sequence for the *msbB* gene is shown in FIG.
15 1. The *tet* gene in the *msbB1::tet* gene confers resistance to 5 mg/L of tetracycline. The resulting strain thus obtained was YS1643.

Strain YS1643 was initially sensitive to 10 mM ethylene glycol bis((b-aminoethyl ether)-N,N,N',N'-tetraacetic
20 acid (EGTA), spontaneously reverted to a EGTA-resistant phenotype. One such revertant, denoted YS1644, was selected by plating YS1643 on EGTA (2mM in Luria agar).

Strain YS1644 was then transduced with the *msbB2*(Δ) *bla sacB* chromosomal element. This transduction process
25 brought in a second version of the disrupted *msbB* gene, denoted as *msbB2*(Δ) as well as the *bla* and *sacB* genes. The *bla* gene is responsible for the transcription of the enzyme β -lactamase, which metabolizes ampicillin, and was subsequently used to select transductants. The *sacB* gene is
30 responsible for the conversion of sucrose into a toxic chemical, levan, that is lethal to the host cells, and was used to select for recombinants. The presence of the *bla* and *sacB* genes allowed the selection of the *amp*^r and *suc*^s strain (denoted as strain YS1645), which contained both the
35 *msbB1::tet* and *msbB2*(Δ) genes.

Strain YS1645 was plated on Luria-Bertani (LB) sucrose to select a *suc*^r *amp*^s *tet*^s derivative to remove the

msbB::tet gene and restore antibiotic sensitivity (i.e., a derivative with deletion of *msbB1::tet bla sacB*). This derivative was denoted as strain YS1646.

In summary YS1646 has deletion mutations in *purI*, 5 and *msbB*. It is also *tet^s*, *amp^s*, and *EGTA^r*.

8.3 INHIBITION OF TUMOR GROWTH WITH YS1646 STRAIN

Intravenous (IV) administration of YS1646, an
10 attenuated strain of *Salmonella typhimurium*, resulted in selective replication within tumors, and concomitant inhibition of tumor growth (see FIG. 17 and Table IV).

In all instances, a staged tumor model was used in which tumors were allowed to become established following
15 tumor cell inoculation and prior to YS1646 administration. As a result of the ability of YS1646 to replicate within the tumor, a shallow dose-response relationship over the effective dose range was determined whereby the extent of tumor inhibition, exerted by low doses of YS1646, approached
20 the level of tumor inhibition achieved at higher doses. This suggested that, even at low doses, significant clinical efficacy could be achieved as long as the bacteria reached the tumor and accumulated within the tumor. Doses below 1×10^2 cfu/mouse gave inconsistent results, possibly due to
25 competition between the ability of YS1646 to reach and colonize the tumor vs. the ability of the animals to clear YS1646.

The efficacy of YS1646 was evaluated in mice previously implanted with B16-F10 melanoma. In this study a single IV
30 dose of YS1646 at 10^4 , 10^5 or 10^6 cfu/mouse significantly reduced tumor size when compared to control treatment, and the degree of tumor size reduction was dose-related. The efficacy observed with the highest dose of YS1646 was superior to that with the positive control, CYTOXAN™ (also
35 known as cyclophosphamide), whereas the efficacy with the mid-dose of YS1646 was equivalent to that with, CYTOXAN™. It is

important to note that the efficacy induced by YS1646 was induced by a single IV dose, whereas that induced by CYTOXAN™ was multiple IV doses (given weekly, for 3 weeks). The ability of YS1646 to inhibit tumor growth, as a function of dose, was examined over an administered dose range of 1×10^4 to 1×10^6 cfu/mouse. Each dosage group was comprised of 10 tumor-bearing animals, which were randomized prior to bacteria administration. Mice were administered bacteria on Day 7, and tumor volumes were measured on Days 10, 13, 17, 20, and 24. For comparison, CYTOXAN™ (cyclophosphamide) was administered once per week at a dose of 200 mg/kg, beginning on Day 7 as well. Mean tumor volumes of each group on Day 24 are presented in Table IV.

15

Table IV.

20

Inoculum Dose (cfu/mouse)	Mean Tumor Volume (mm ³) ±S.D.	T/C	Percent Inhibition
0	4728±804	-	0
10 ⁴	1011±375	0.214	78
10 ⁵	560±176	0.118	88
10 ⁶	279±91	0.059	94

The differences observed between individual groups were deemed significant when analyzed either by the Wilcoxon signed rank test analysis, or by a two-tailed t-test. As indicated in Table IV, increasing tumor inhibition was observed with increasing dose of YS1646. All doses were found to give significant antitumor activity (T/C of less than an equal to 42%), as defined by the Drug Evaluation Branch of the Division of Cancer Treatment, National Cancer Institute (Bethesda, MD) (Vendetti, J.M., Preclinical drug evaluation: rationale and methods, Semin. Oncol. 8:349-361; 1981), and doses of 1×10^5 cfu/mouse gave results equivalent to or better than cyclophosphamide. A linear correlation between YS1646 dose and tumor inhibition was not observed due to the ability of YS1646 to replicate preferentially within

the tumor, which led to greater than expected potency at lower doses. Intravenous administration of YS1646, an attenuated strain of *Salmonella typhimurium*, resulted in selective replication within tumors, and concomitant inhibition of tumor growth. Between inoculum doses of 1×10^4 to 1×10^6 cfu/mouse, a dose-response for inhibition of tumor growth was obtained, ranging from 78% to 94% inhibition of tumor growth. At the two highest inoculum doses, the level of tumor growth inhibition was comparable to or better than that achieved by optimal treatment with cyclophosphamide.

8.4 VIRULENCE

At a dose of 1×10^6 cfu/mouse, YS1646 does not cause lethality, in contrast to the parental wide type strain ATCC 14028, which causes 100% mortality at a dose of 1×10^2 cfu/mouse. This indicates that YS1646 is greater than 10,000-fold less virulent than the parental wild type strain. The antitumor efficacy was observed at doses of 10^4 to 10^6 cfu/mouse, whereas lethality was not observed until the doses were $>10^6$ cfu/mouse. The dose inducing mortality was 1 to 100-fold greater than the dose inducing anti-tumor efficacy (see FIG. 18).

8.5 ANTIBIOTIC SUPPRESSION OF YS1646 INDUCED MORTALITY FOLLOWING LETHAL INFECTION

25

The ability of ampicillin and ciprofloxacin to suppress infection by YS1646 was evaluated by determining the ability of antibiotics to prevent mortality in C57BL/6 mice inoculated with 5×10^6 cfu (LD_{50} equivalent).

30

Groups were divided into the following treatment categories: 1) untreated control, 2) ampicillin-treated, 3) ciprofloxacin-treated, and 4) ciprofloxacin and ampicillin treated. Antibiotic treatment was initiated 3 days following bacteria administration and animals were observed daily for appearance and mortality for 14 days. Results presented herein demonstrate that use of antibiotic was able to suppress

mortality following lethal bacterial infections (see FIG. 18).

9. DEPOSIT OF MICROORGANISMS

5 The following microorganisms were deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110-2209, on September 9, 1997, and have been assigned the indicated Accession numbers:

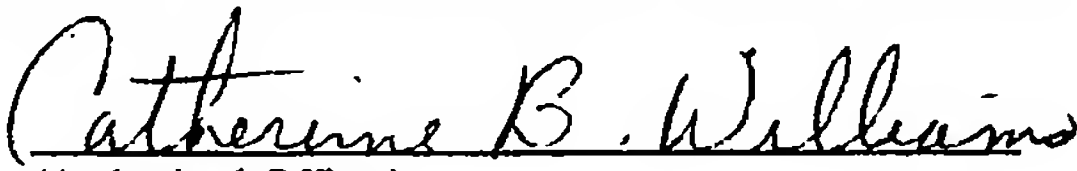
10	<u>Microorganism</u>	<u>ATCC Accession No.</u>
	YS8211	202026
	YS1629	202025
	YS1170	202024

15 The following microorganisms were deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110-2209, on 25 August, 1998, and have been assigned the indicated Accession numbers:

20	<u>Microorganism</u>	<u>ATCC Accession No.</u>
	YS1646	202165
	YS1456	202164

The invention claimed and described herein is not
25 to be limited in scope by the specific embodiments, including but not limited to the deposited microorganism embodiments, herein disclosed since these embodiments are intended as illustrations of several aspects of the invention. Indeed, various modifications of the invention in addition to those
30 shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

A number of references are cited herein, the
35 entire disclosures of which are incorporated herein, in their entirety, by reference.

MICROORGANISMS	
Optional Sheet in connection with the microorganism referred to on page <u>45</u> , lines <u>10-22</u> of the description *	
A. IDENTIFICATION OF DEPOSIT * Further deposits are identified on an additional sheet *	
Name of depositary institution * American Type Culture Collection	
Address of depositary institution (including postal code and country) * 10801 University Blvd. Manassas, VA 20110-2209 US	
Date of deposit * <u>September 9, 1997</u> Accession Number * <u>202026</u>	
B. ADDITIONAL INDICATIONS * (leave blank if not applicable). This information is continued on a separate attached sheet	
C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE * (if the indications are not all designated States)	
D. SEPARATE FURNISHING OF INDICATIONS * (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")	
E. <input checked="" type="checkbox"/> This sheet was received with the International application when filed (to be checked by the receiving Office)	
<div style="text-align: right;"> (Authorized Officer)</div>	
<input type="checkbox"/> The date of receipt (from the applicant) by the International Bureau *	
was _____ (Authorized Officer)	

Form PCT/RO/134 (January 1981)

International Application No: PCT/ /

Form PCT/RO/134 (cont.)

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US

<u>Accession No.</u>	<u>Date of Deposit</u>
202025	September 9, 1997
202024	September 9, 1997
202165	August 25, 1998
202164	August 25, 1998

WHAT IS CLAIMED IS:

1. A mutant *Salmonella* sp. comprising a genetically modified *msbB* gene in which the mutant *Salmonella* is capable of targeting a solid tumor when administered *in vivo*.
2. The mutant *Salmonella* of claim 1 which is designated YS1629 and having ATCC Accession No. 202025 or is designated YS1170 and having ATCC Accession No. 202024 or is designated YS8211 and having ATCC Accession No. 202026.
3. The mutant *Salmonella* of claim 1 which is selected from the group consisting of *Salmonella typhi*, *Salmonella choleraesuis*, and *Salmonella enteritidis*.
4. The mutant *Salmonella* of claim 1 which expresses an altered lipid A molecule.
5. The mutant *Salmonella* of claim 1 which induces TNF α expression at about 5 percent to about 40 percent of that induced by a wild type *Salmonella* sp.
6. The mutant *Salmonella* of claim 1 which induces TNF α expression at about 10 percent to about 35 percent of that induced by a wild type *Salmonella* sp.
7. Lipopolysaccharide purified from the mutant *Salmonella* of claim 1 which induces TNF α expression at less than or equal to 0.001 percent of that induced by a wild type *Salmonella* sp.
8. The mutant *Salmonella* of claim 1 in which a chelating agent inhibits growth by about 90 percent compared to the growth of a wild type *Salmonella* sp.

9. The mutant *Salmonella* of claim 1 in which a chelating agent inhibits growth by about 99 percent compared to the growth of a wild type *Salmonella* sp.

5 10. The mutant *Salmonella* of claim 1 in which a chelating agent inhibits growth greater than 99 percent compared to the growth of a wild type *Salmonella* sp.

10 11. The mutant *Salmonella* of claim 8, 9, or 10 in which the chelating agent is selected from the group consisting of Ethylenediaminetetraacetic Acid (EDTA), Ethylene Glycol-bis(β -aminoethyl Ether) N, N, N', N',-Tetraacetic Acid (EGTA) and sodium citrate.

15 12. The mutant *Salmonella* of claim 1 which survives in macrophages at about 50 percent to about 30 percent of the level of survival of a wild type *Salmonella* sp.

20 13. The mutant *Salmonella* of claim 1 which survives in macrophages at about 30 percent to about 10 percent of the level of survival of a wild type *Salmonella* sp.

25 14. The mutant *Salmonella* of claim 1 which survives in macrophages at about 10 percent to about 1 percent of the level of survival of a wild type *Salmonella* sp.

30 15. A method of inhibiting growth or reducing volume of a solid tumor cancer, comprising administering an effective amount of the mutant *Salmonella* sp. of claim 1 to a patient having a solid tumor cancer.

35 16. The method according to claim 15 in which the mutant *Salmonella* is selected from the group consisting of

Salmonella typhi, *Salmonella choleraesuis*, and *Salmonella enteritidis*.

17. The method according to claim 15 in which the mutant *Salmonella* expresses an altered lipid A molecule.

18. The method according to claim 15 in which the mutant *Salmonella* induces TNF α expression at about 5 percent to about 40 percent of that induced by a wild-type *Salmonella* sp.

19. The method according to claim 15 in which the mutant *Salmonella* induces TNF α expression at about 10 percent to about 35 percent of that induced by a wild-type *Salmonella* sp.

20. The method according to claim 15 in which lipopolysaccharide purified from the mutant *Salmonella* induces TNF α expression at less than or equal to 0.001 percent of that induced by a wild type *Salmonella* sp.

21. The method according to claim 15 in which a chelating agent inhibits growth of the mutant *Salmonella* by about 90 percent compared to the growth of a wild-type *Salmonella* sp.

22. The method according to claim 15 in which a chelating agent inhibits growth of the mutant *Salmonella* by about 99 percent compared to the growth of a wild-type *Salmonella* sp.

23. The method according to claim 15 in which a chelating agent inhibits growth of the mutant *Salmonella* by greater than 99 percent compared to the growth of a wild-type *Salmonella* sp.

24. The method according to claim 21, 22 or 23 in which the chelating agent is selected from the group consisting of EDTA, EGTA and sodium citrate.

5 25. The method according to claim 15 in which the mutant *Salmonella* survives in macrophages at about 50 percent to about 30 percent of the level of survival of a wild-type *Salmonella* sp.

10 26. The method according to claim 15 in which the mutant *Salmonella* survives in macrophages at about 30 percent to about 10 percent of the level of survival of a wild-type *Salmonella* sp.

15 27. The method according to claim 15 in which the mutant *Salmonella* survives in macrophages at about 10 percent to about 1 percent of the level of survival of a wild-type *Salmonella* sp.

20 28. The method according to claim 15 in which the solid tumor cancer is melanoma.

29. The method according to claim 15 in which the solid tumor cancer is colon carcinoma.

25

30. The method according to claim 15 in which the solid tumor cancer is selected from the group consisting of lung cancer, liver cancer, kidney cancer, prostate cancer, and breast cancer.

30

31. A pharmaceutical composition comprising an amount of the mutant *Salmonella* of claim 1 effective to inhibit growth or reduce volume of a solid tumor cancer; and a pharmaceutically acceptable carrier.

35

32. A mutant *Salmonella* sp. comprising a genetically modified *msbB* gene and a genetically modified

purI gene in which the mutant *Salmonella sp.* is capable of targeting a solid tumor when administered *in vivo*.

33. A mutant *Salmonella sp.* of claim 32 in which
5 the genetic modifications are deletion mutations.

34. The mutant *Salmonella sp.* of claim 32 which is designated YS1646 and having ATCC Accession No. 202165 or is designated YS1456 and having the ATCC Accession No. 202164.

10

35. A mutant *Salmonella sp.* comprising a genetically modified *msbB* gene and a genetically modified biosynthetic pathway gene in which the biosynthetic pathway mutation confers attenuated virulence.

15

36. A method of inhibiting growth or reducing volume of a solid tumor cancer, comprising administering an effective amount of the mutant *Salmonella sp.* of claim 32 to a patient having a solid tumor cancer.

20

37. An improved method for selecting genetic alterations of a bacterium, wherein the improvement comprises selecting a phenotypic variant which, when grown on a medium containing sucrose, produces colonies in which the edges of
25 the mutant colonies are fuzzy or rough.

30

35

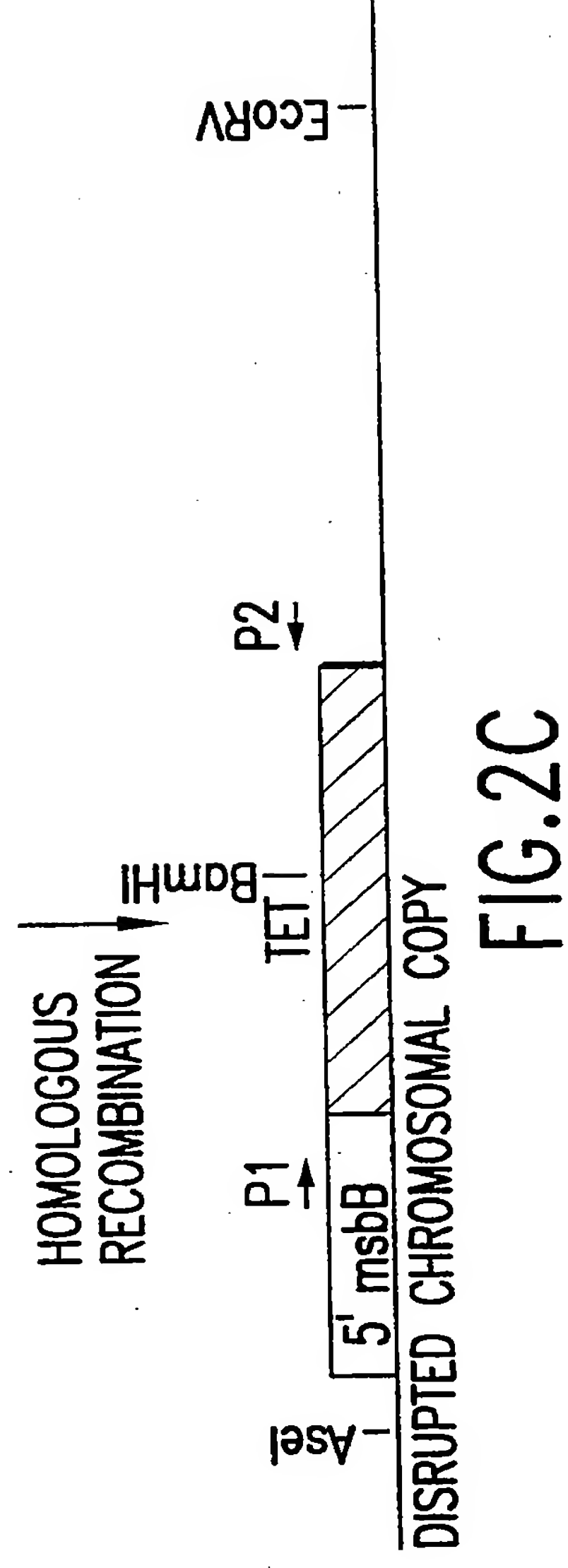
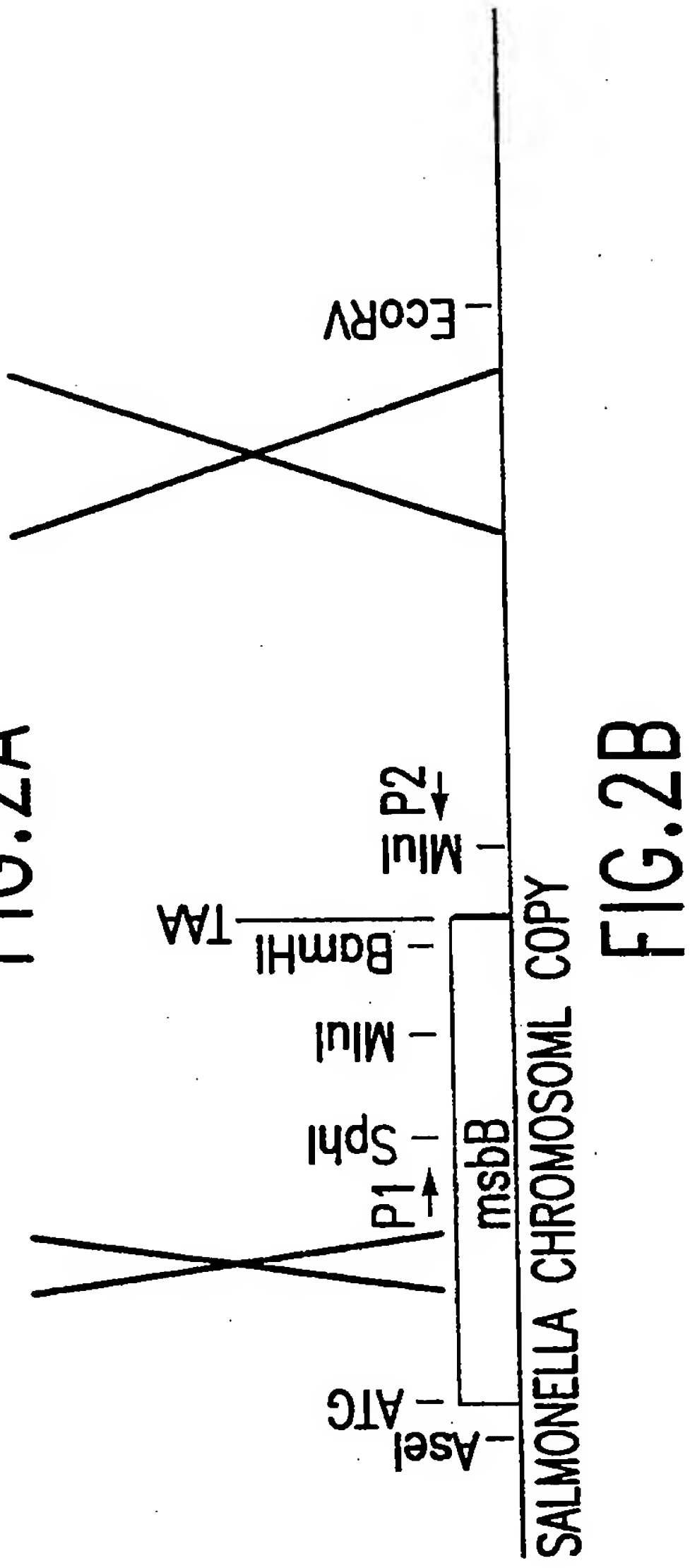
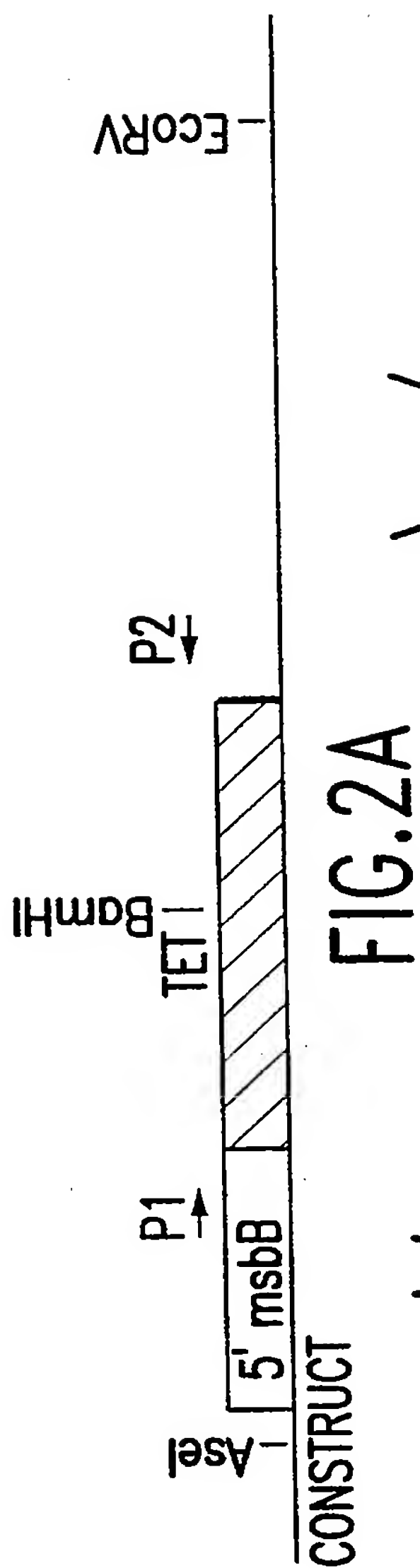
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M E T K K N N S E

FIG.1A

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G T G T A T A T G C T A A A C C G A T C C C T G A C T A C C A G A T T C G C C C G C C A T G G A C C A T C G C T A C G G C T G A C G A C C A C A C T A T C G C C A G A
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FIG.1B



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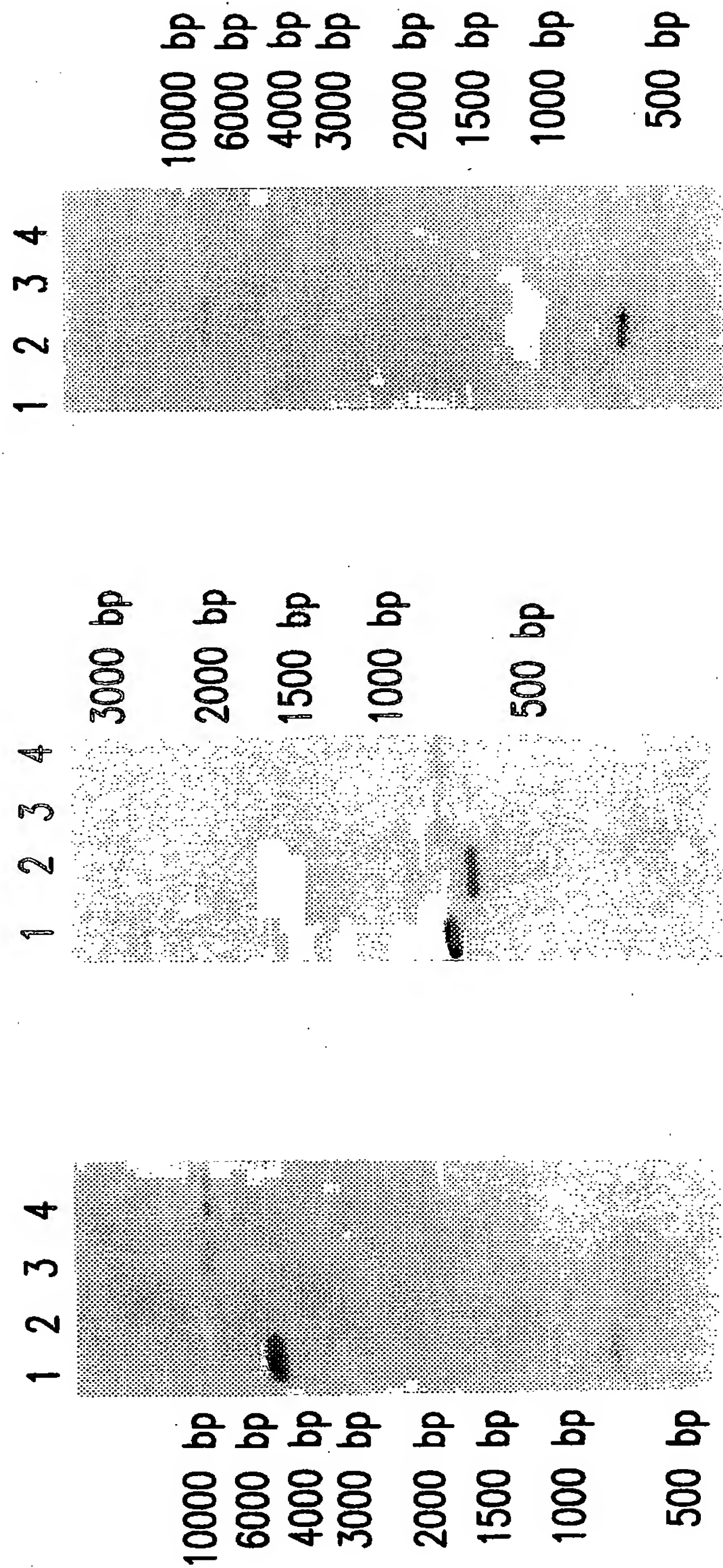


FIG. 3C

FIG. 3B

FIG. 3A

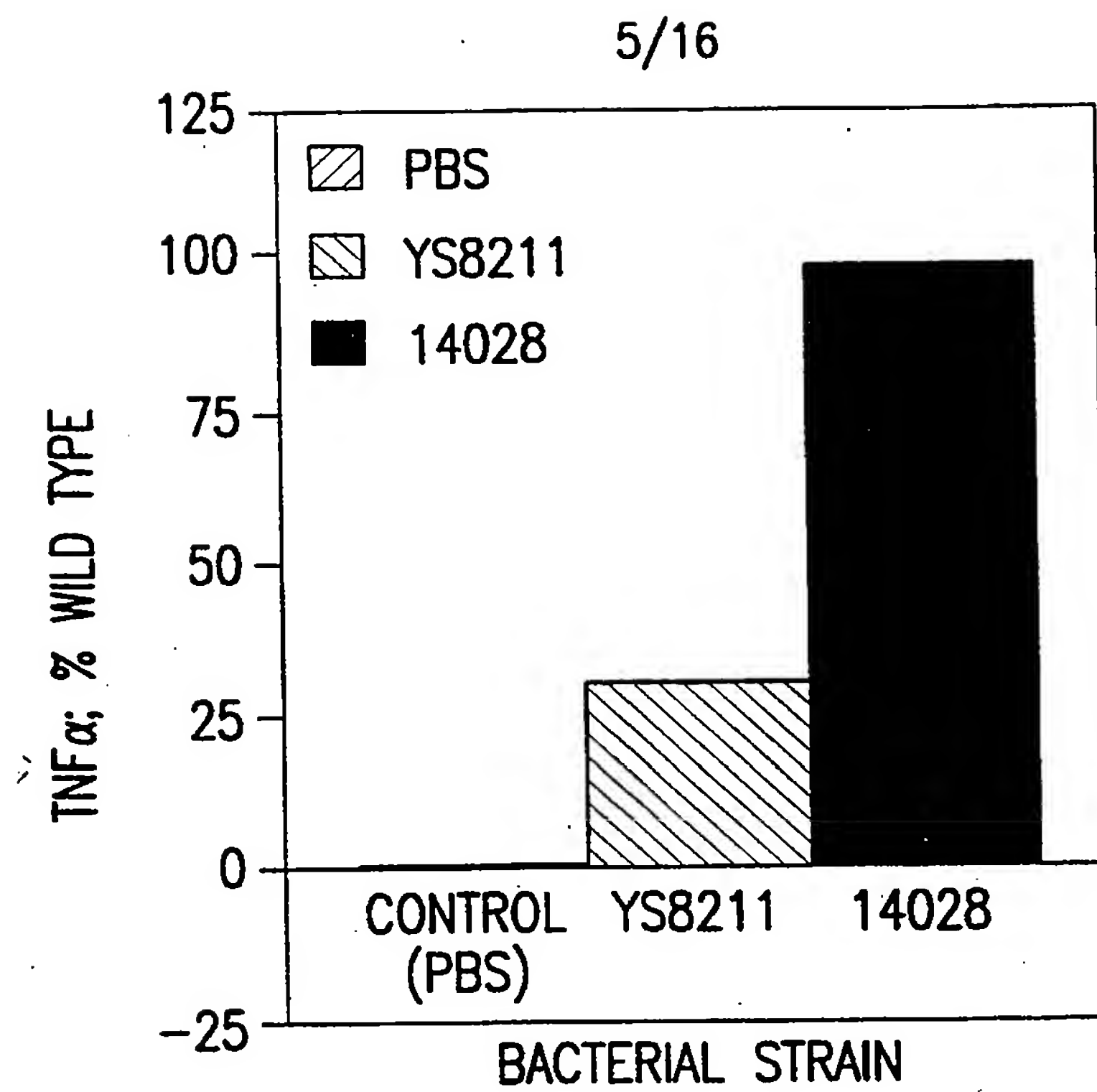
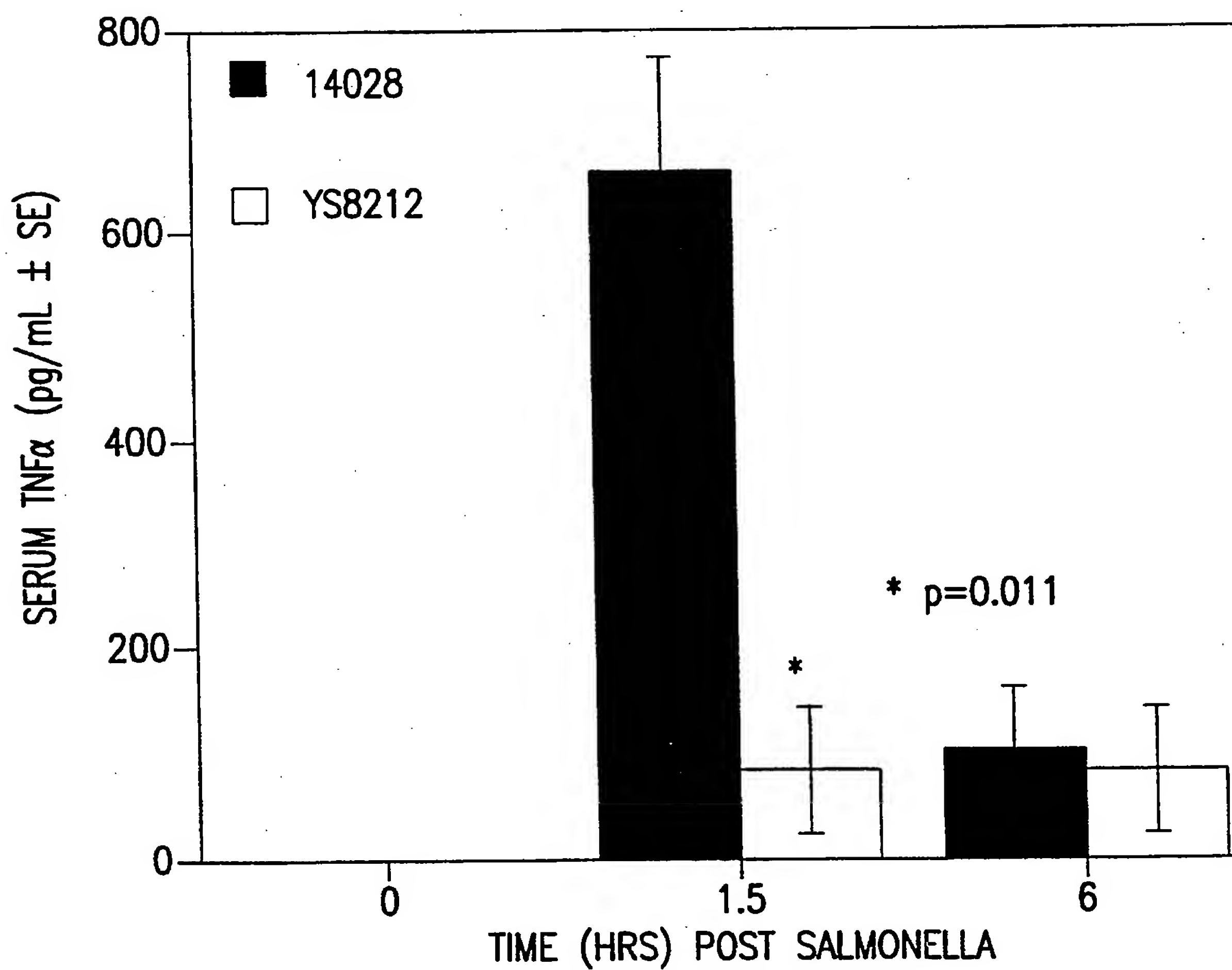


FIG.4



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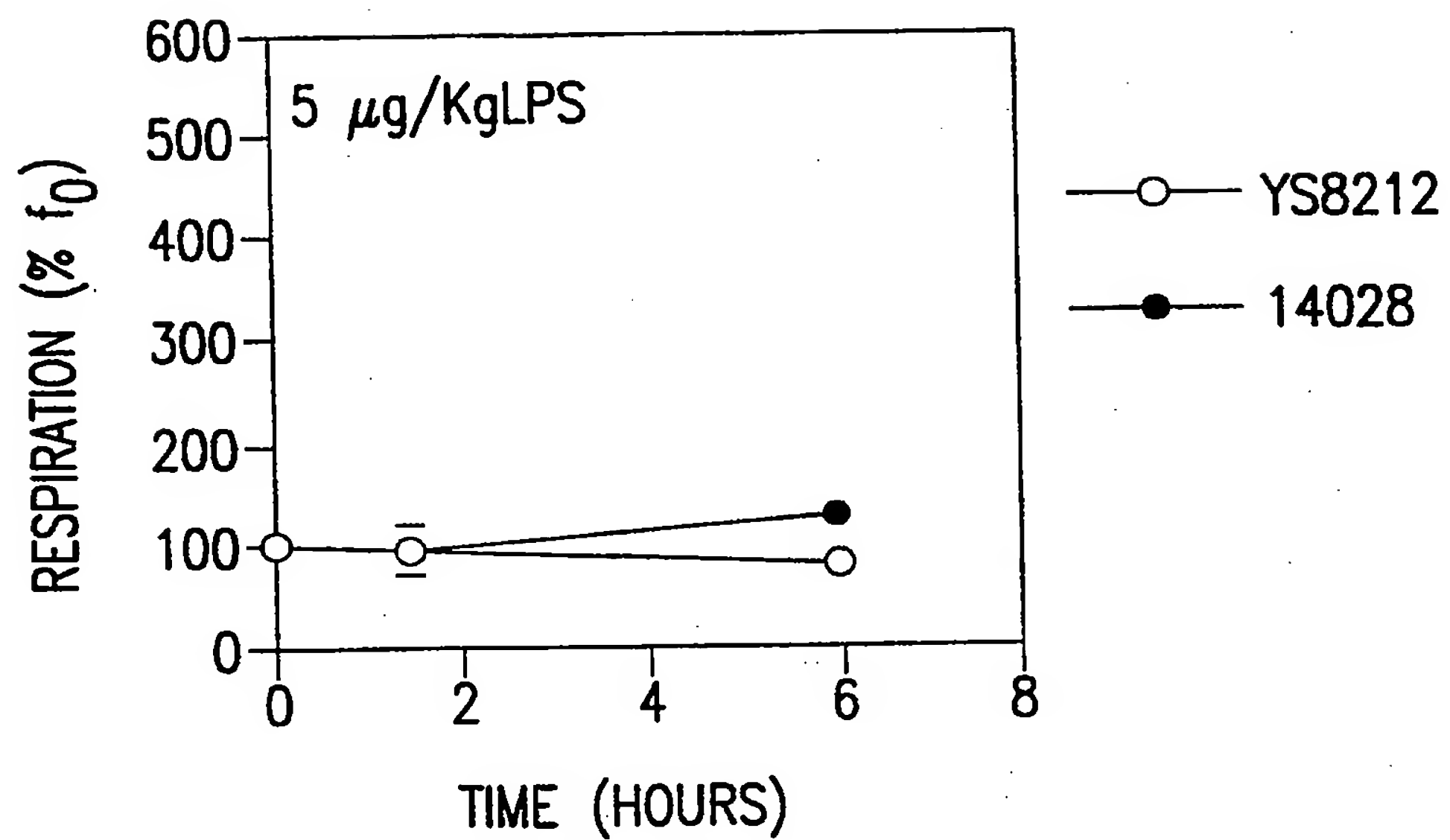


FIG. 6A

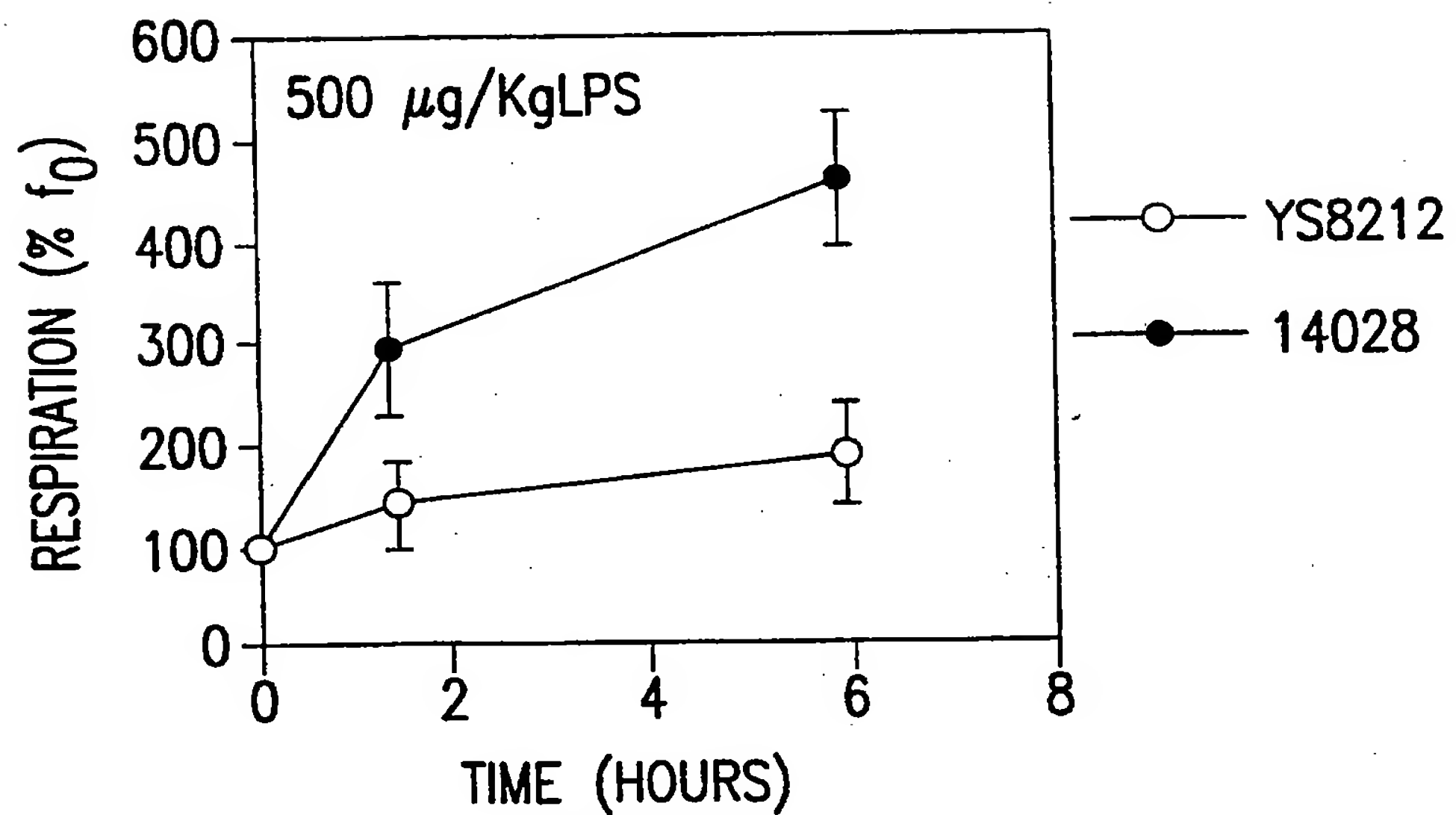


FIG. 6B

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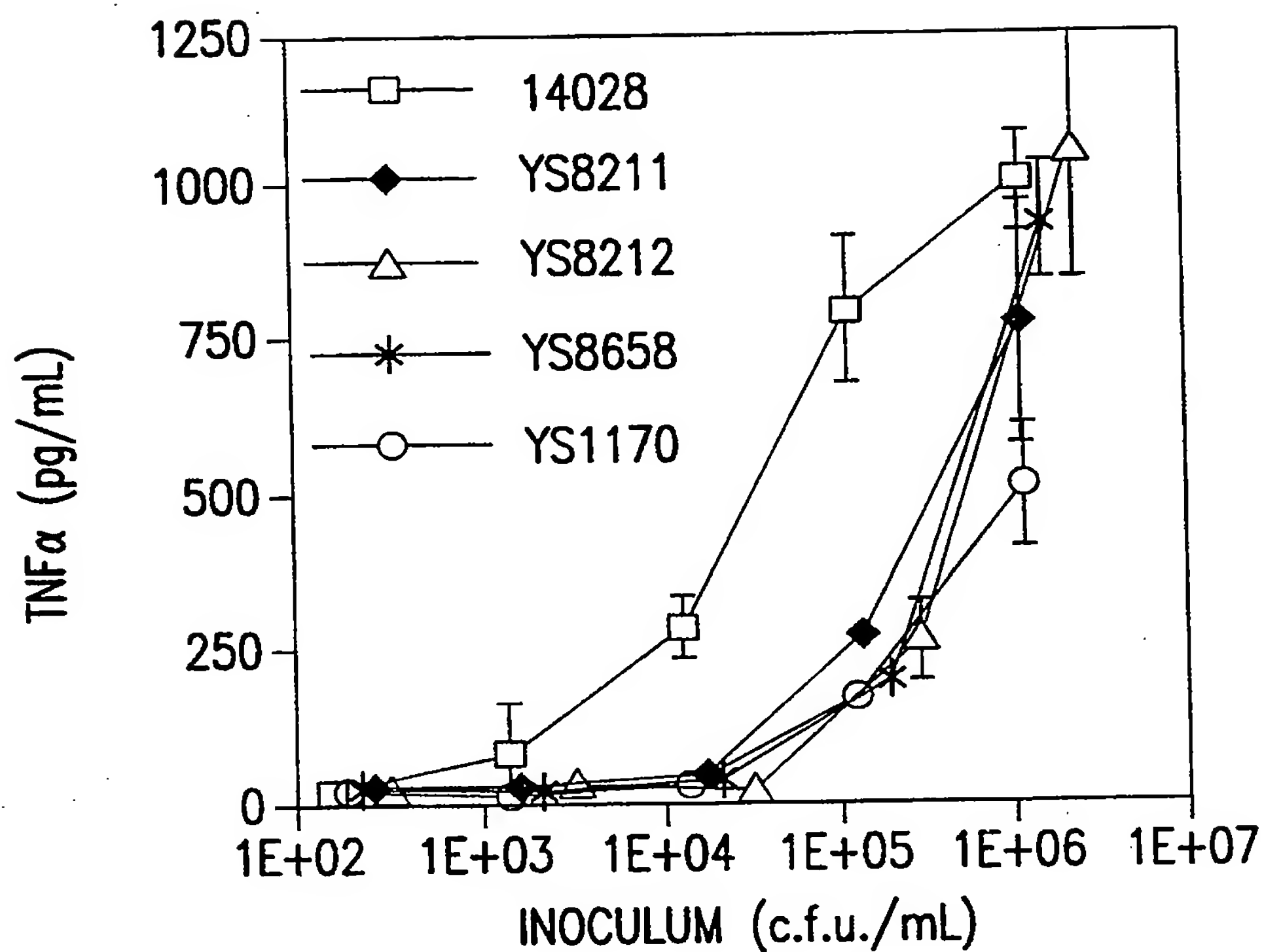
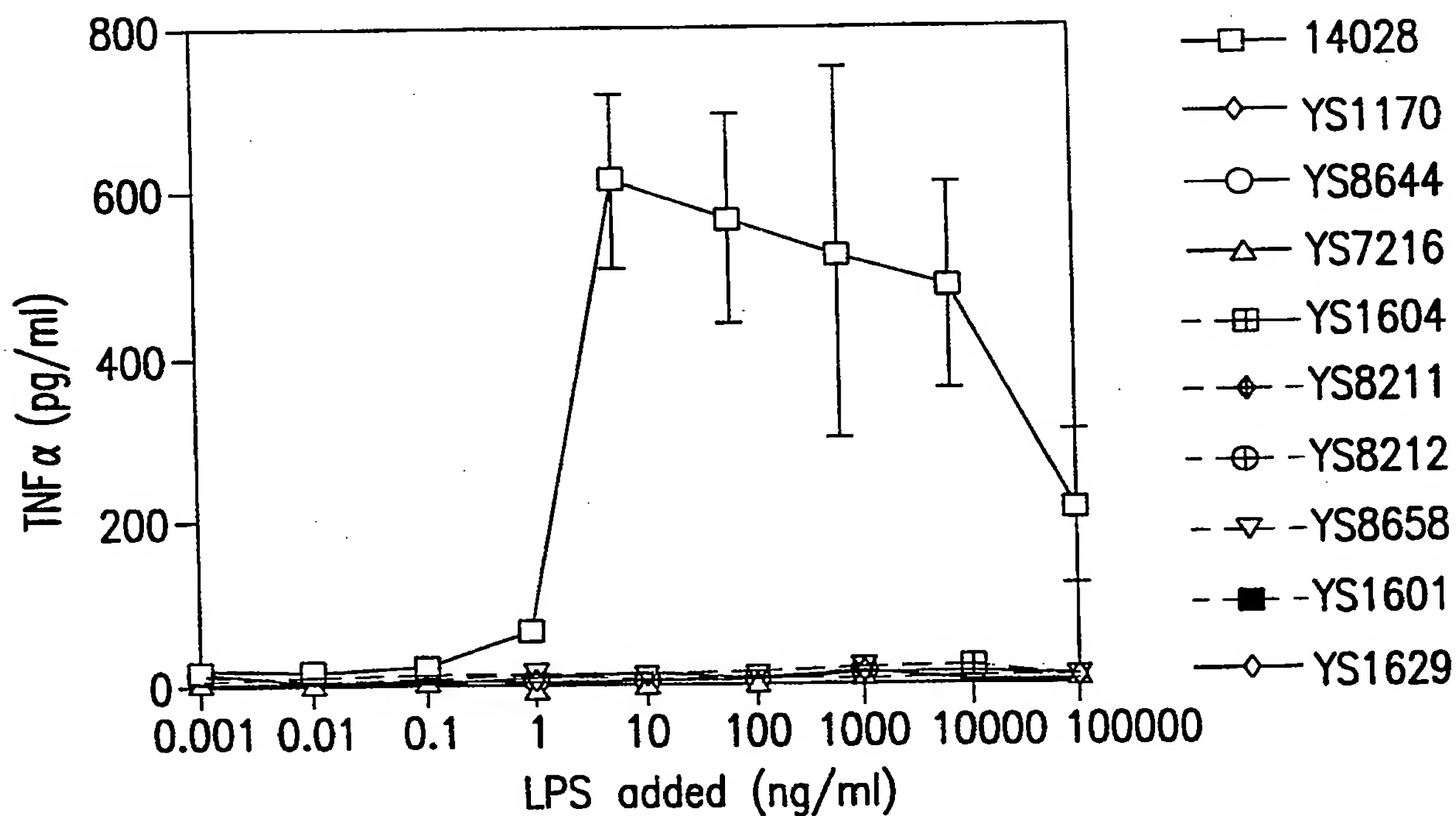


FIG.7



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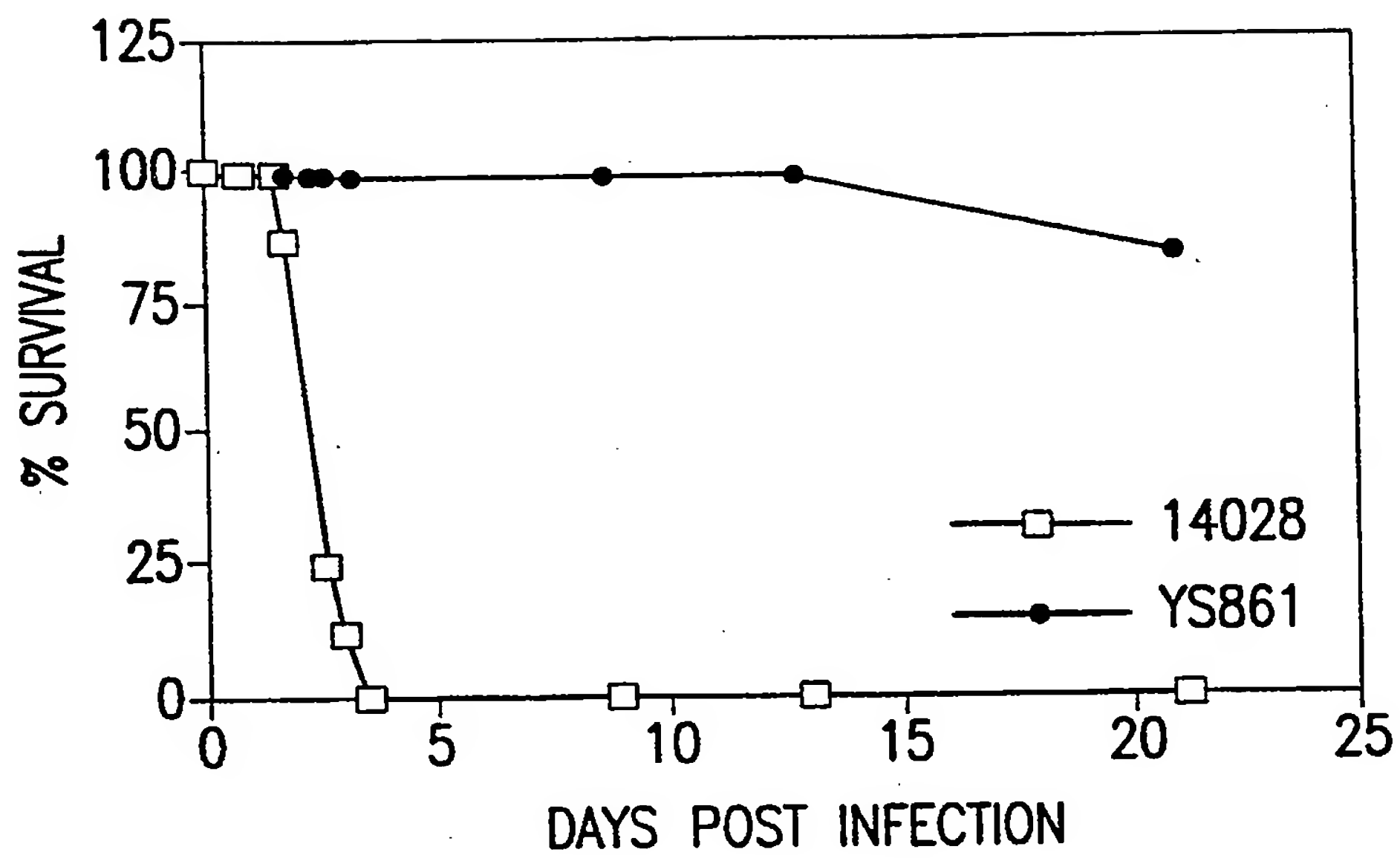


FIG.9A

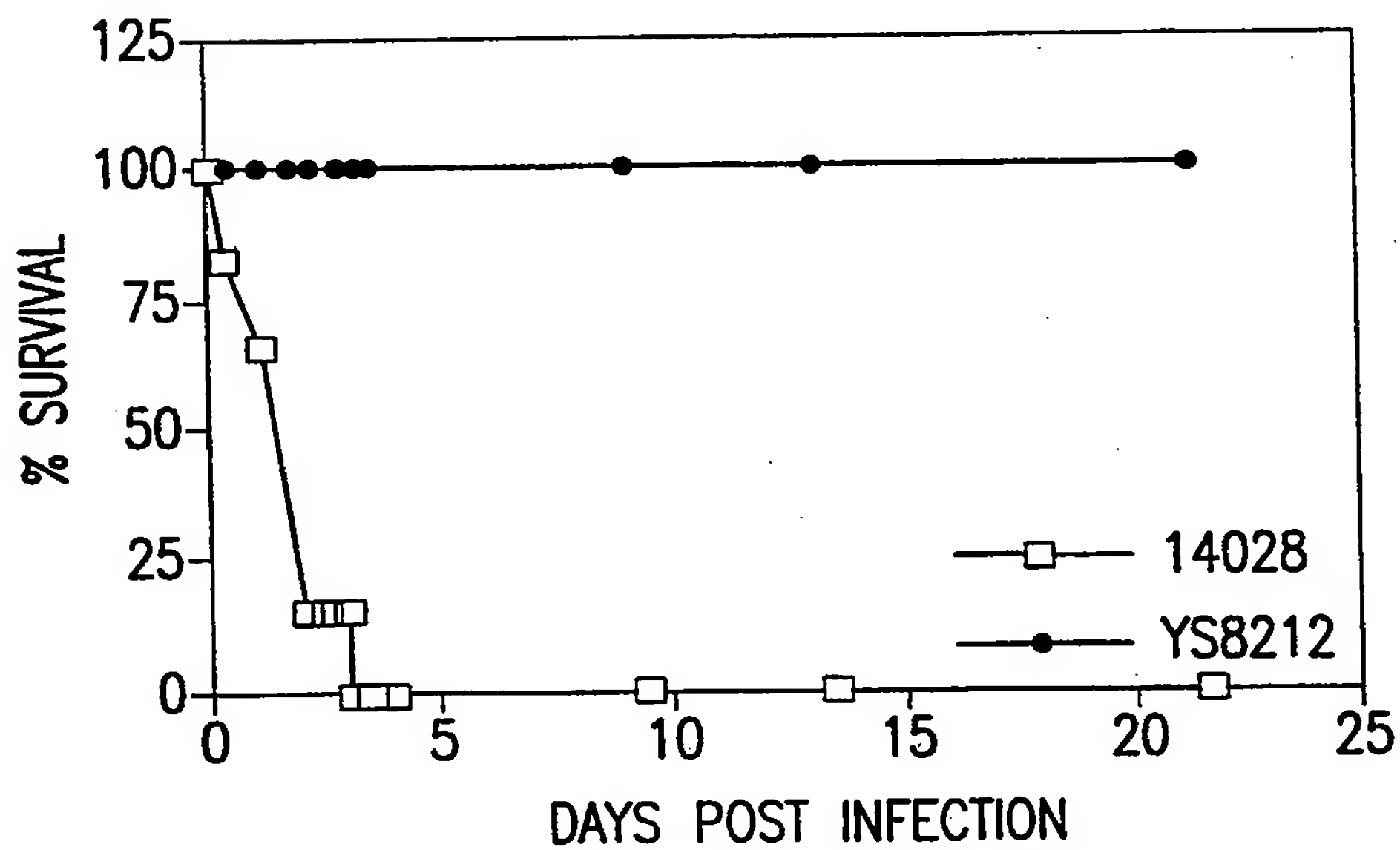


FIG.9B

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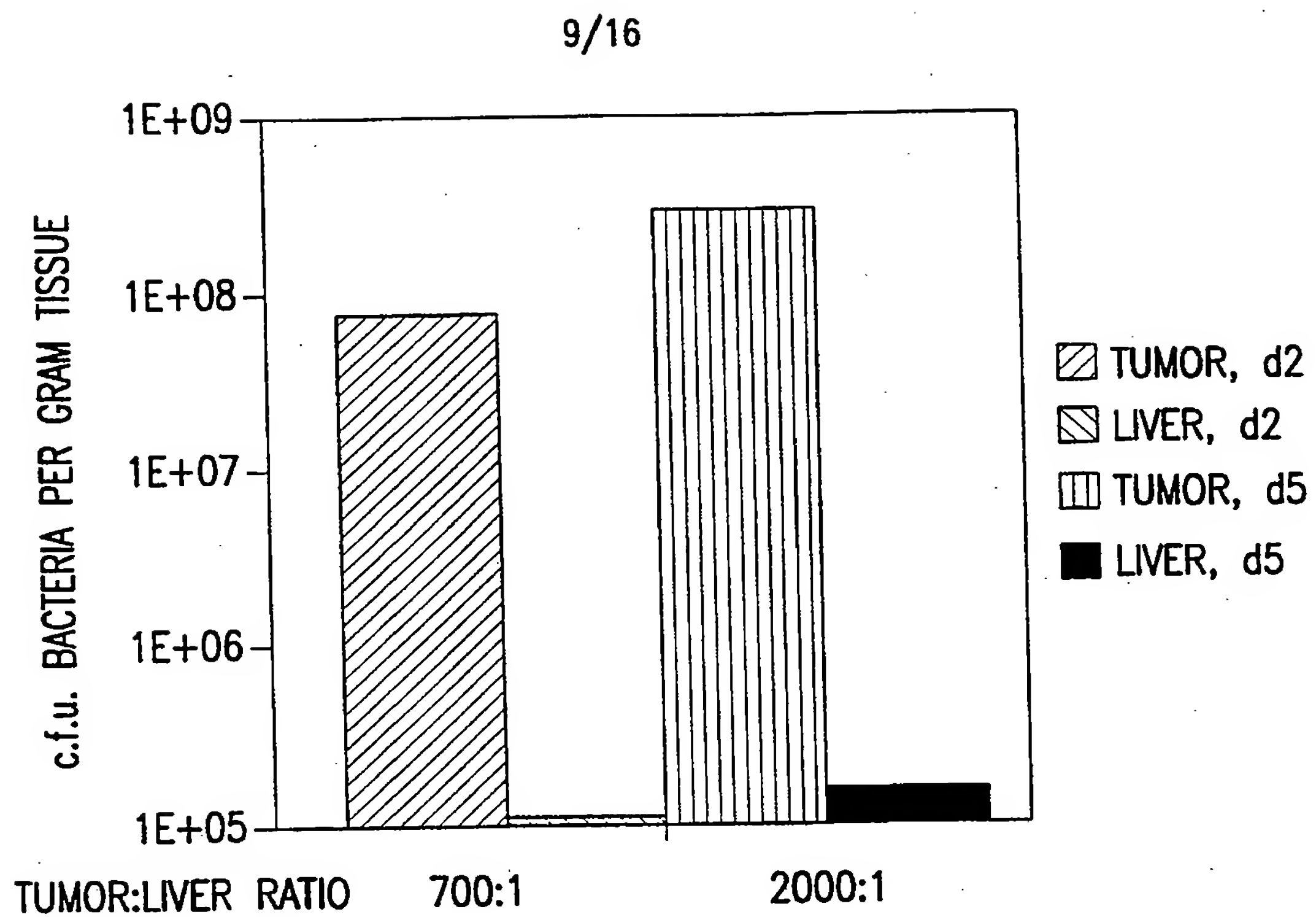


FIG.10

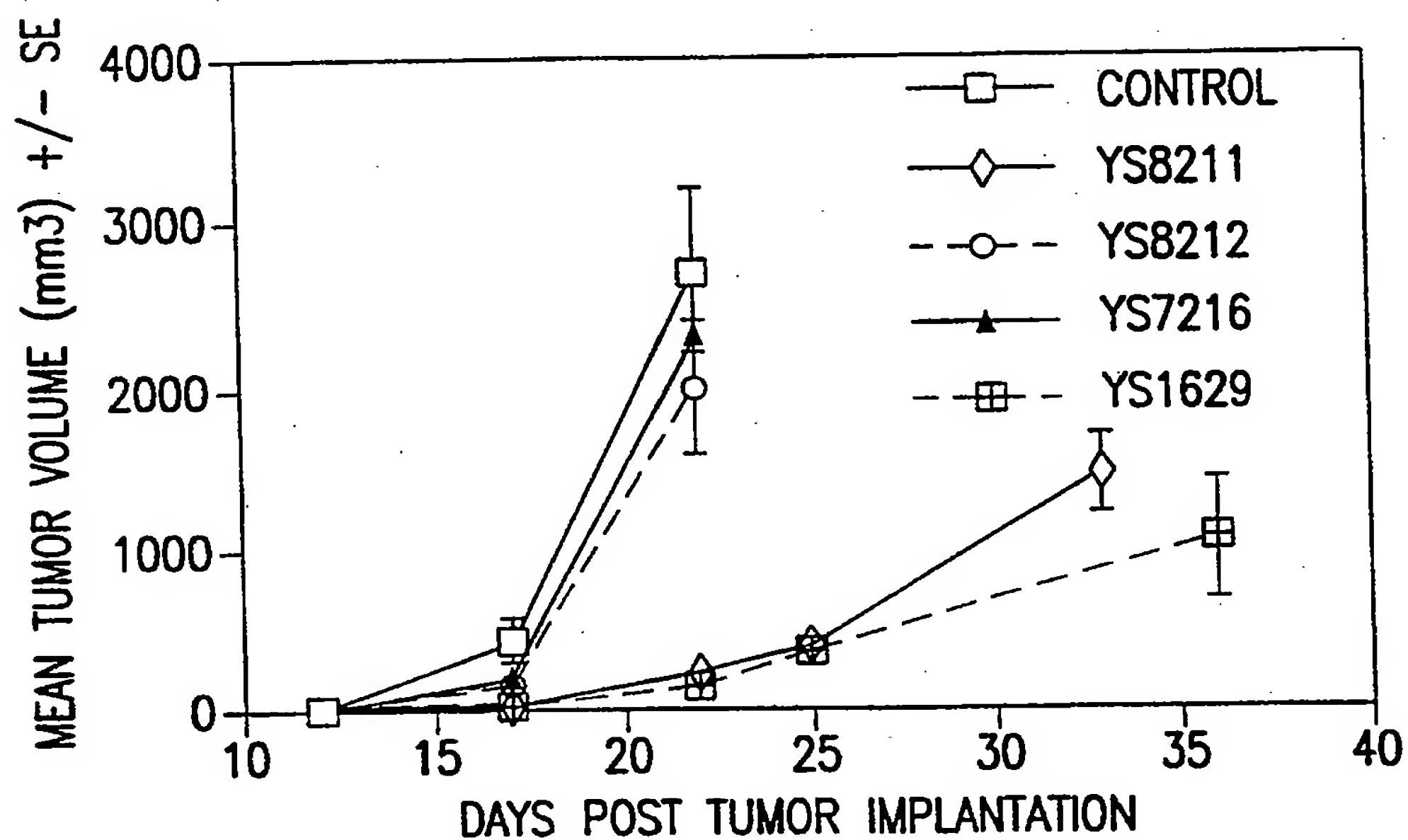


FIG.11

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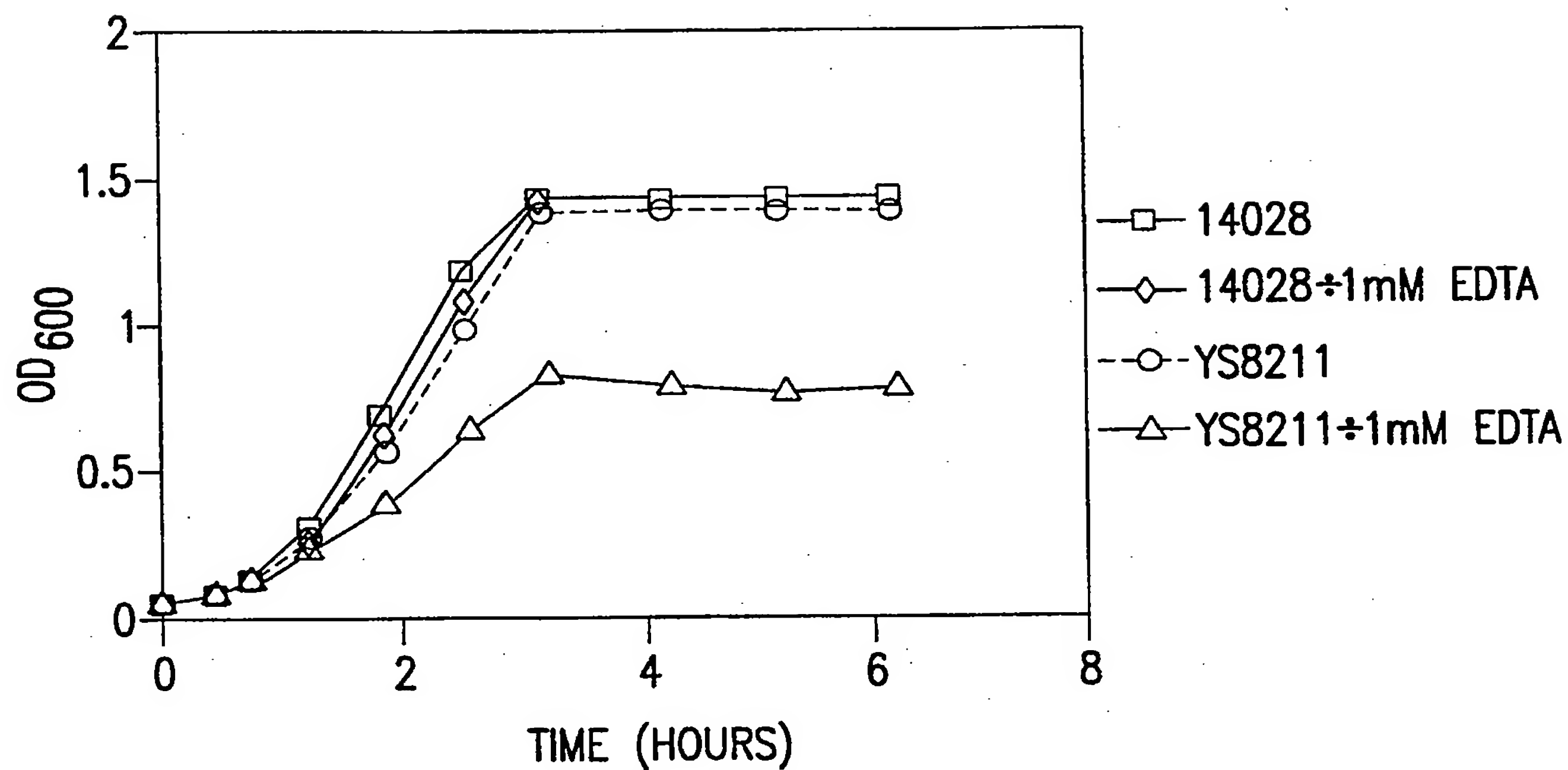


FIG.12A

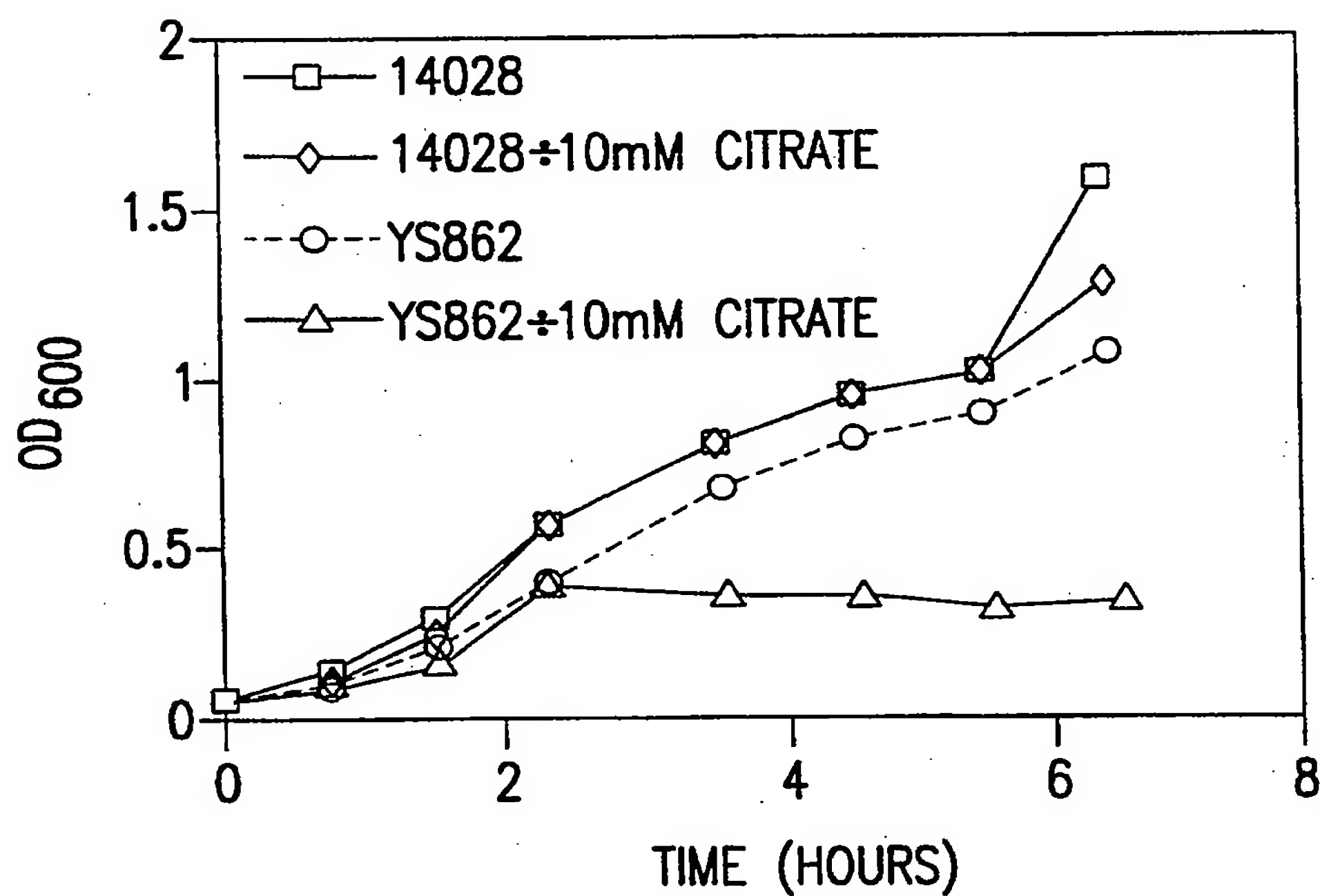


FIG.12B

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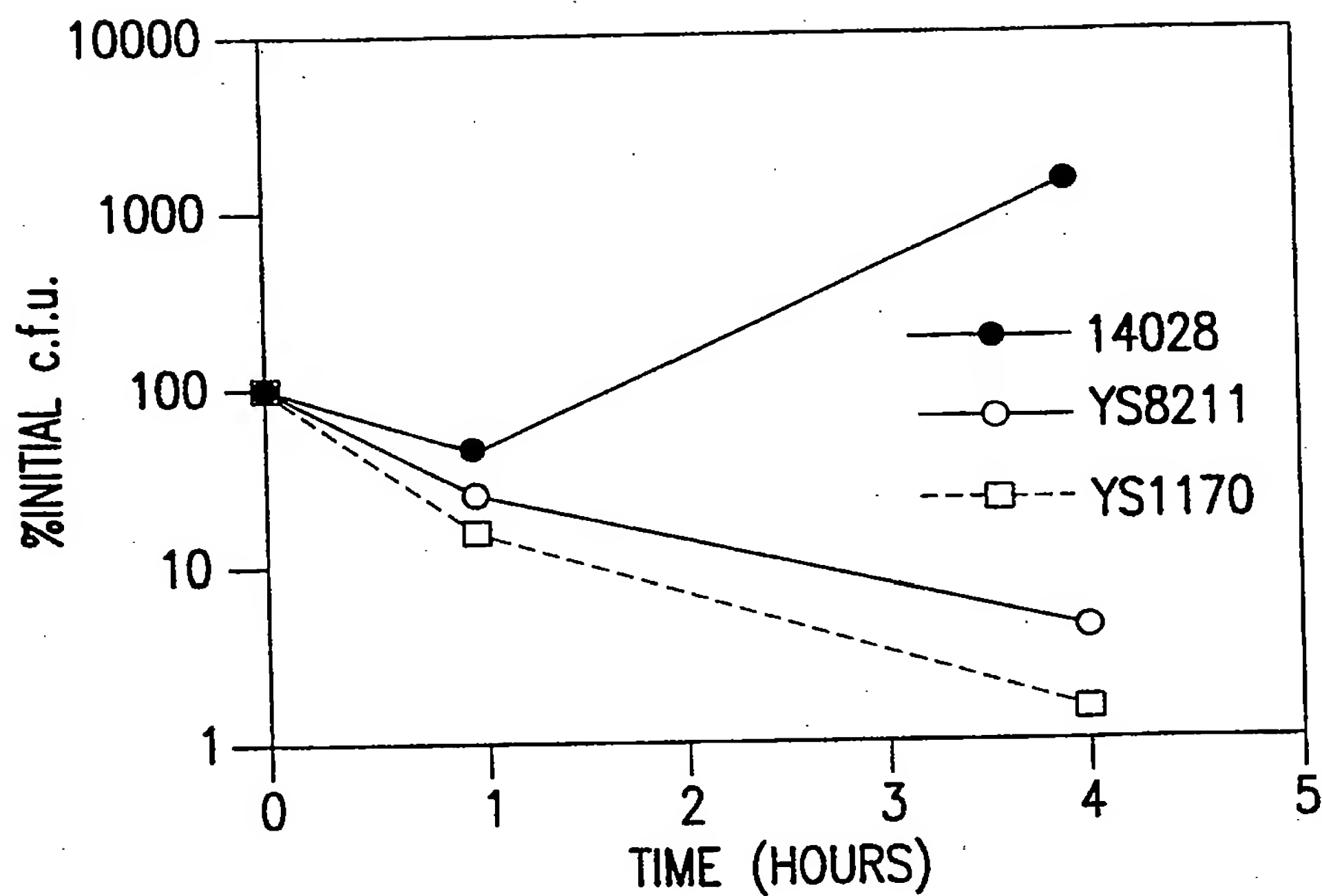


FIG. 13A

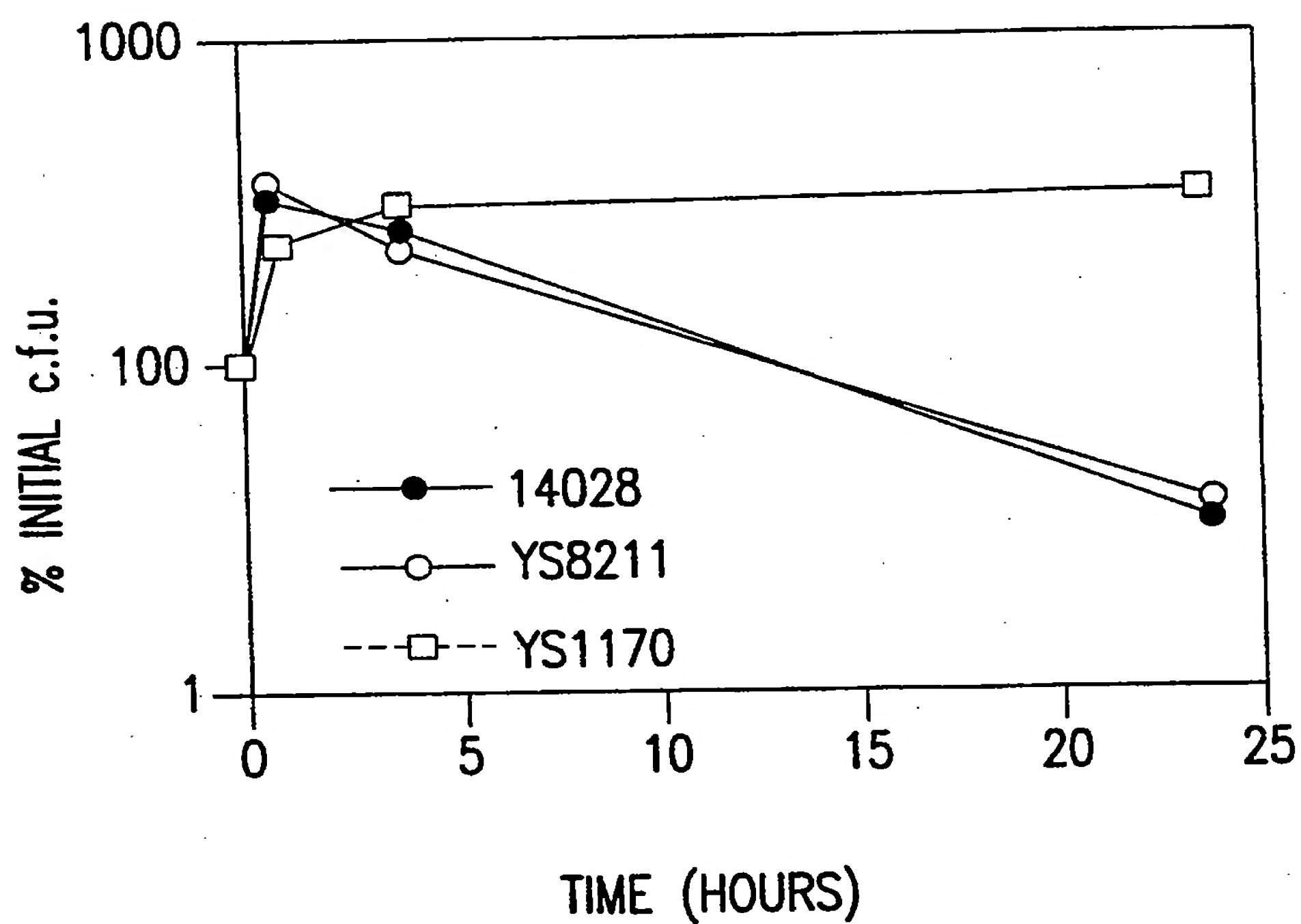


FIG. 13B

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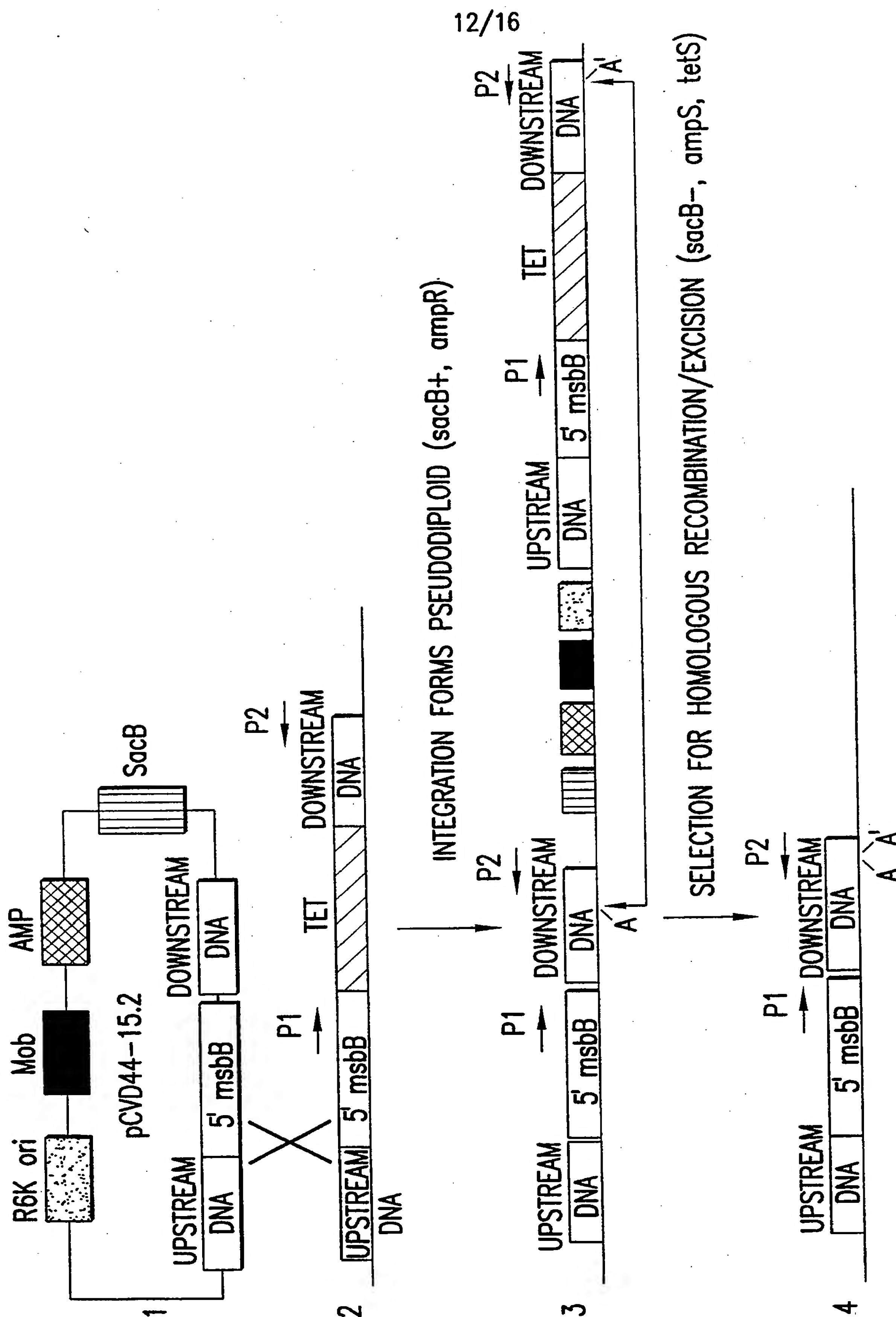


FIG. 14

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DERIVATION OF STRAIN YS1456

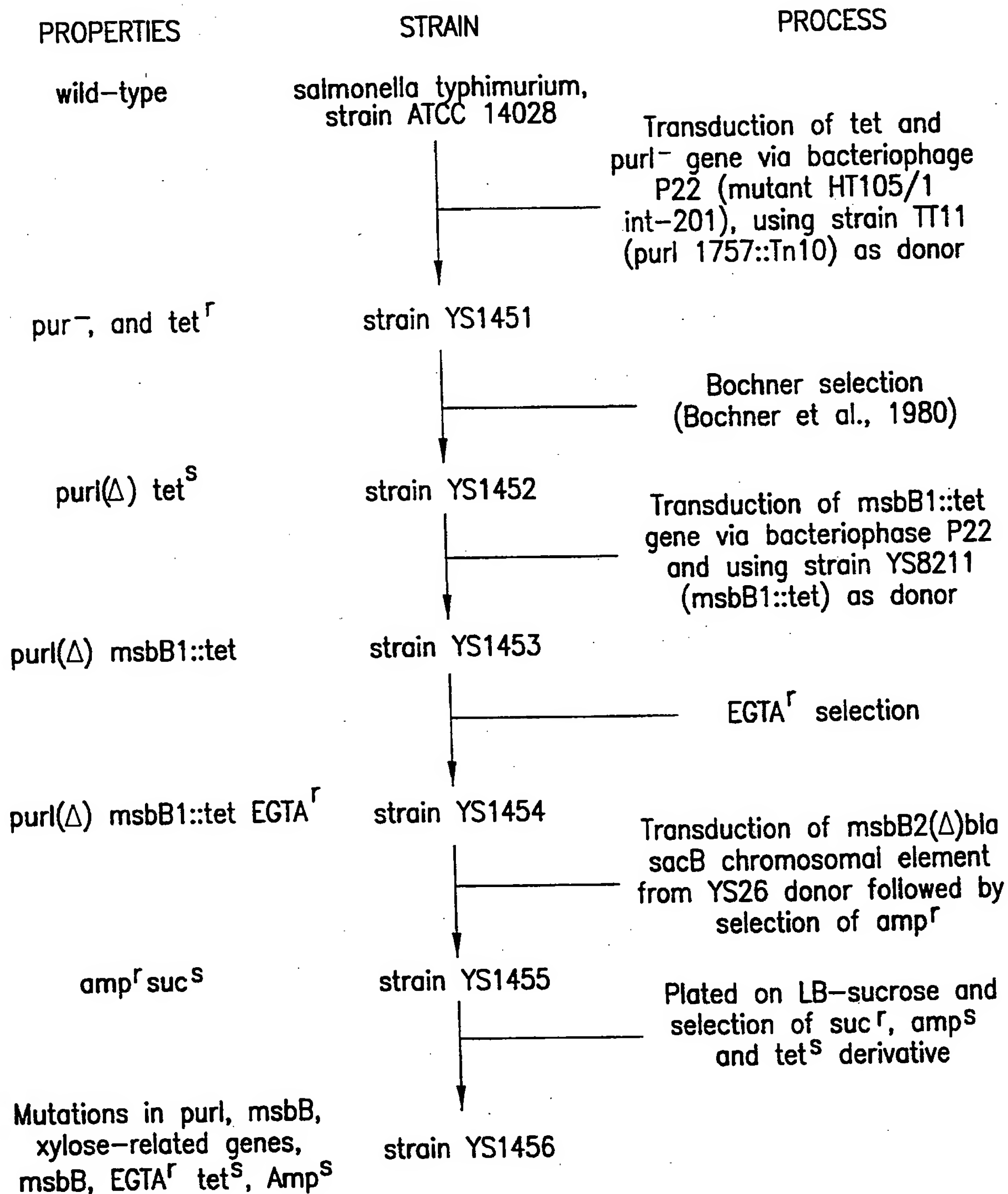


FIG.15

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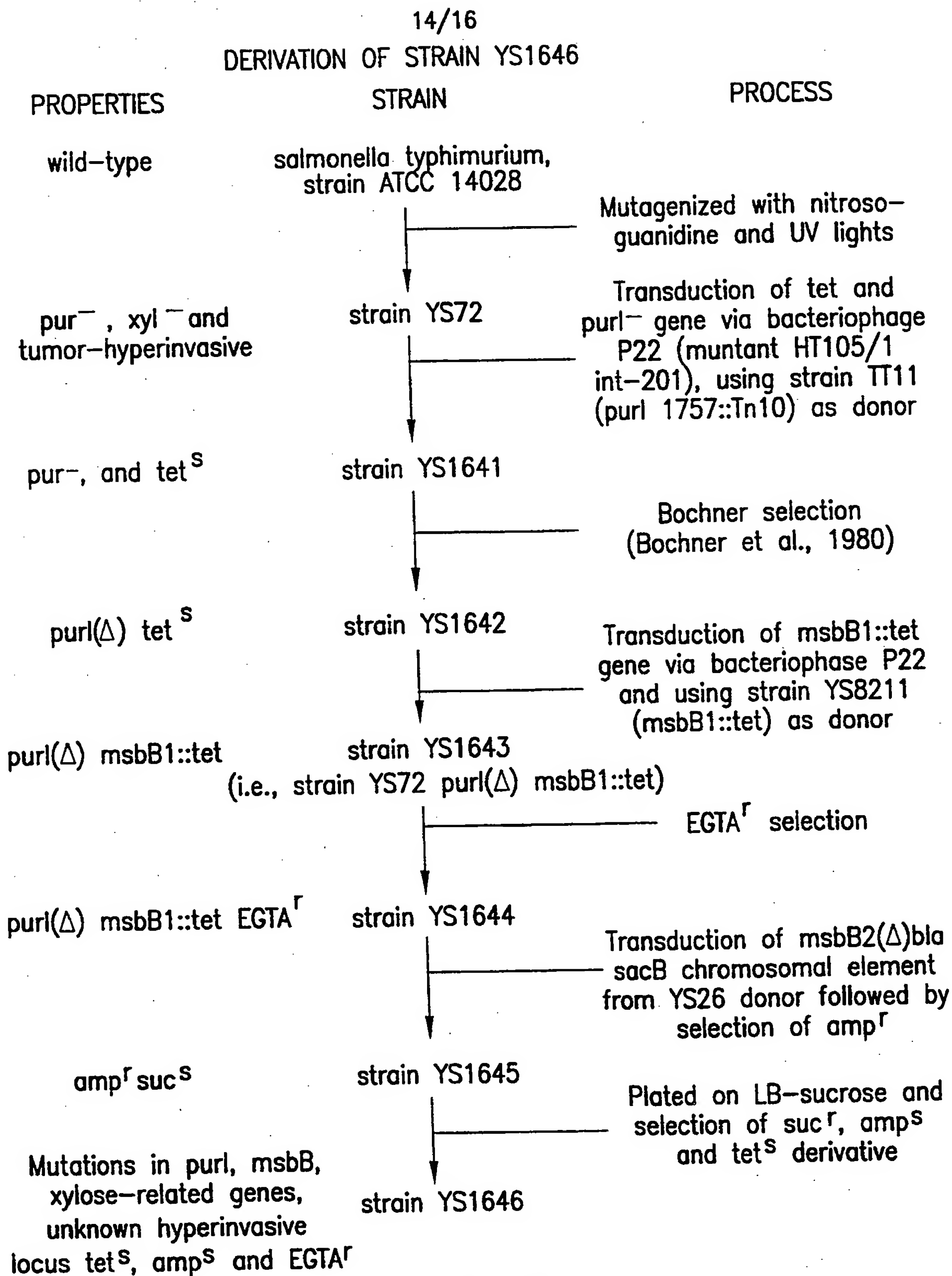


FIG.16

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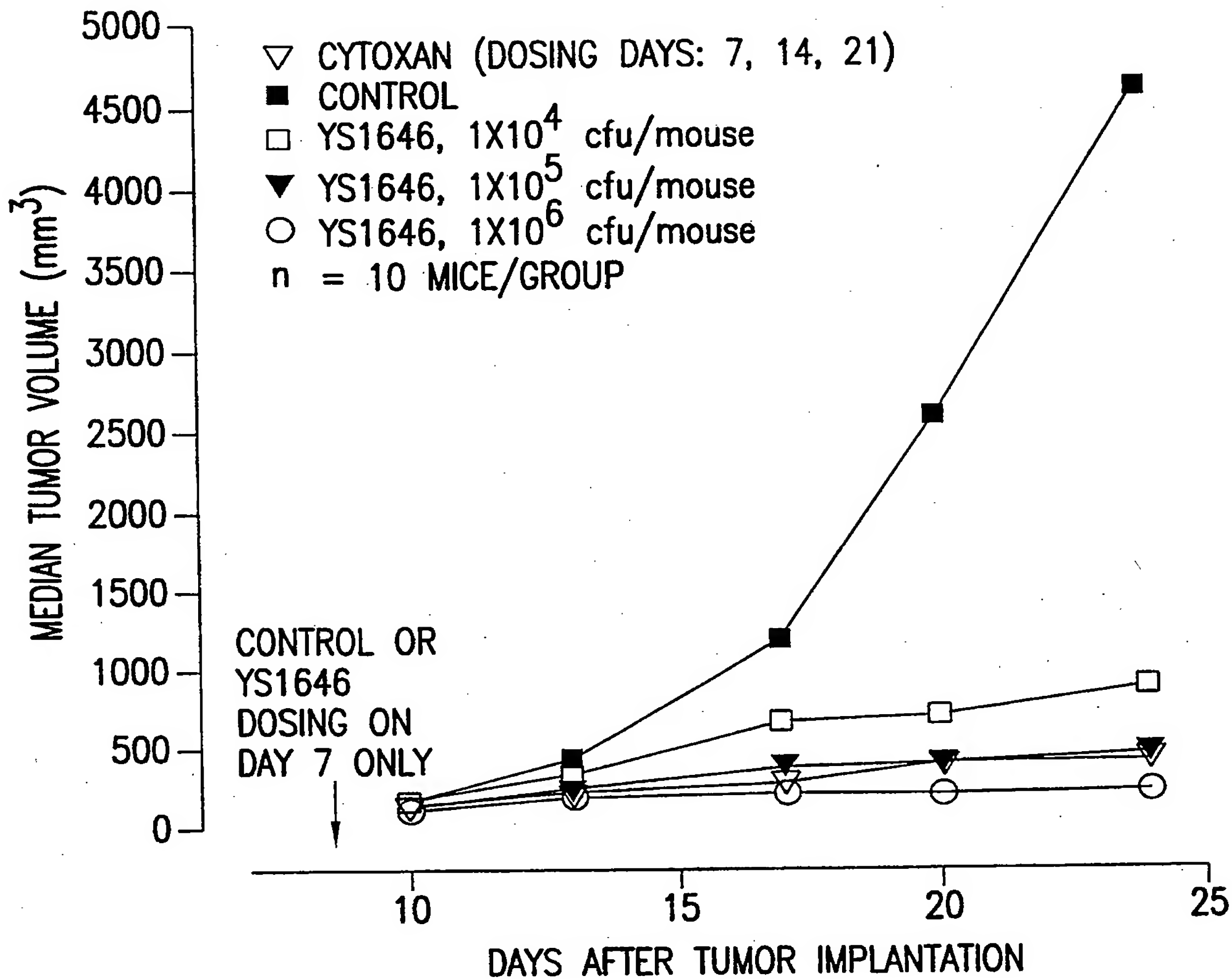


FIG.17

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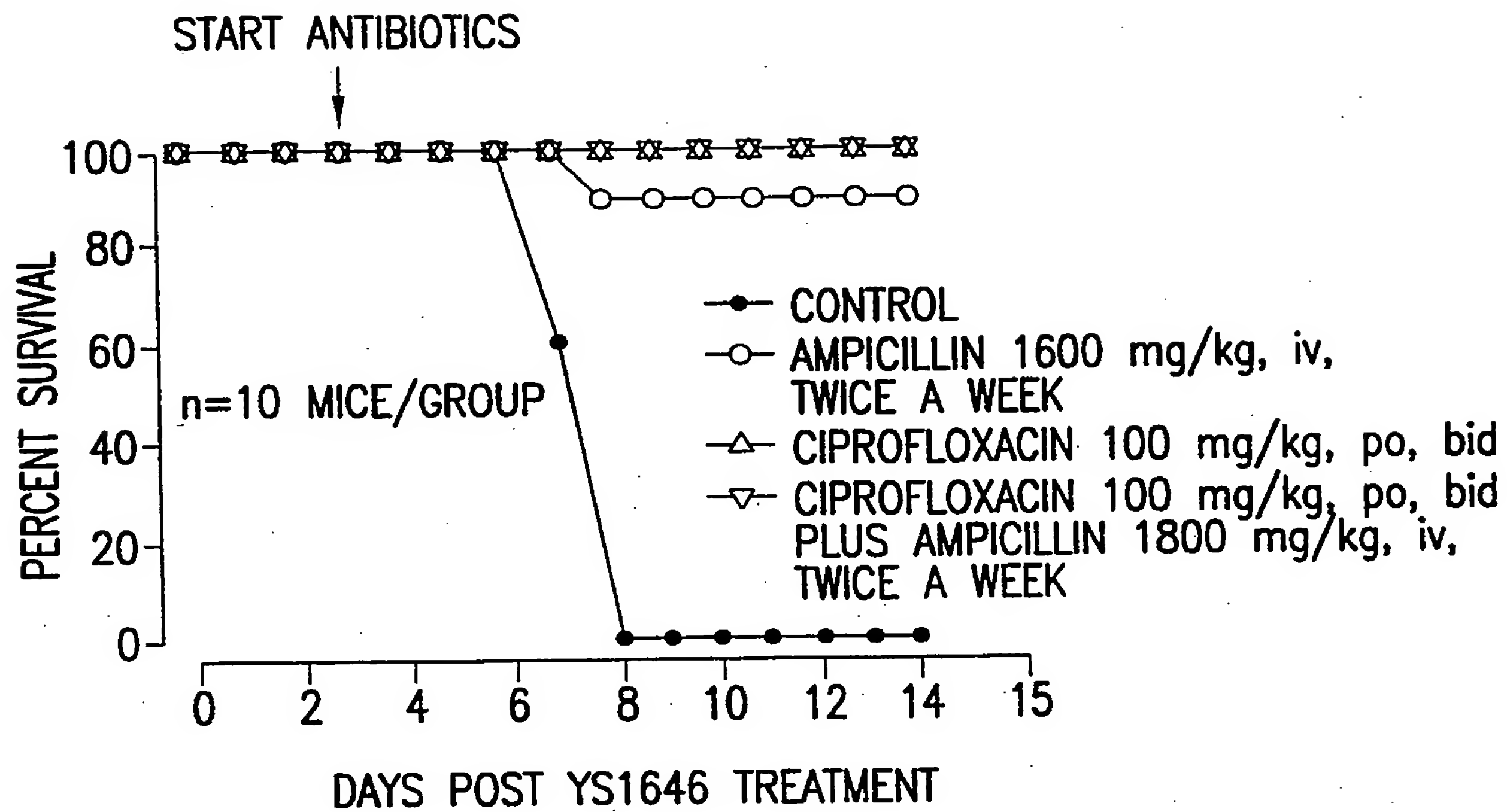


FIG.18

SEQUENCE LISTING

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YALE UNIVERSITY

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REDUCED VIRULENCE

<130> 8002-042

<140> PCT/US98/

<141> 1998-09-09

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Ala Thr Leu Gly Arg Phe Ala Gly Arg Leu Gly Lys Ser Ser Arg Arg
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Arg Ala Leu Ile Asn Leu Ser Leu Cys Phe Pro Gln Arg Ser Glu Ala
 65 70 75 80

Glu Arg Glu Ala Ile Val Asp Glu Met Phe Ala Thr Ala Pro Gln Ala
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Met Ala Met Met Ala Glu Leu Ala Met Arg Gly Pro Lys Lys Ile Gln
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Gln Arg Val Asp Trp Glu Gly Leu Glu Ile Ile Glu Glu Met Arg Arg
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Asn Asp Glu Lys Val Ile Phe Leu Val Pro His Gly Trp Gly Val Asp
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Ile Pro Ala Met Leu Met Ala Ser Gln Gly Gln Lys Met Ala Ala Met
 145 150 155 160

Phe His Asn Gln Gly Asn Pro Val Phe Asp Tyr Ile Trp Asn Thr Val
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Arg Arg Arg Phe Gly Gly Arg Leu His Ala Arg Asn Asp Gly Ile Lys
 180 185 190

Pro Phe Ile Gln Ser Val Arg Gln Gly Tyr Trp Gly Tyr Tyr Leu Pro
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Asp Gln Asp His Gly Pro Glu His Ser Glu Phe Val Asp Phe Phe Ala

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Arg Leu Thr Ile Gln Ile Arg Pro Pro Met Asp Asp Leu Leu Thr Ala		
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Asp Asp His Thr Ile Ala Arg Arg Met Asn Glu Glu Val Glu Ile Phe		
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Val Gly Pro His Pro Glu Gln Tyr Thr Trp Ile Leu Lys Leu Leu Lys		
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/18701

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 1/20; A61K 38/14; A01N 37/18

US CL : 435/252.8; 530/322; 514/2+

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/252.8; 530/322; 514/2+

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, CAPLUS, STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	WO 97/18837 A1 (UNIVERSITY OF MARYLAND AT BALTIMORE), 29 May 1997, see pages 24, 25 and entire document.	1, 3-14, 31 ----- 17-27, 29, 30, 32, 36, 37.
X - Y	PAWELEK et al. Tumor-targeted Salmonella as A Novel Anti-melanoma Vector. Melanoma Research, 4th World Conference on Melanoma, Sydney, Australia. 10-14 June 1996, Vol. 7, (Suppl. 1), S141, see entire abstract.	15, 16, 28 ----- 17-27, 29, 30, 32

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

05 NOVEMBER 1998

Date of mailing of the international search report

21 December 1998

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